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# **Improving food safety of sprouts and cold-smoked salmon by physical and biological preservation methods**

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# Chapter 1

## General introduction

In the past decades the consumption of convenient food products has increased dramatically. The consumption of 18 kg of convenience food per capita in Germany in 1985 has more than doubled up to 2005 (Anonymous, 2006). The sales for the sector of fresh-cut produce in the US for example have grown steadily from \$5 billion in 1994, \$6 billion in 1997, \$12 billion in 2003 up to \$15 billion in 2005 (IFPA, 2006). “Ready-to-eat-“(RTE) food belongs to one category of the group of convenience food which is defined as “intended to be eaten as purchased without further preparation by the consumer, particularly without additional cooking (FDA, 2001)”. The most problematic products thereof are those intended to be eaten raw. In general they can also be allotted to the group of minimally processed foods. Rolle and Chism (1997) define minimally processing of fruits and vegetables as operations that include washing, selecting, peeling, slicing etc. and that keep the food as a living tissue. Examples for RTE food of animal origin are seafood (e.g. fish salads, smoked fish), soft cheese and fermented sausages, examples of plant origin are vegetables (e.g. salads, seed sprouts) and fruits.

Based on the fact that in products eaten raw no germ reduction step is commonly applied, the consumer may be exposed to increased risks due to microbial contamination. Among these organisms *Listeria monocytogenes*, salmonellae or enteropathogenic *E. coli* (EHEC) are of primary importance. (Nguyen and Carlin, 1994; FDA 2001; FAO/WHO, 2003).

The ensurance of a continuous hygiene concept “from farm to fork”, based on Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP), is an exclusively means to obtain the product safety to a highest degree.

Due to the current situation mentioned above, it seems necessary to give special attention to the group of RTE foods. This coincides with initiatives of the FDA, WHO, as well as the European Commission (FDA 2001; WHO, 2002; Anonymous, 2002a). Especially in the U.S. it is described in details. Federal food safety regulatory agencies consider the control of listeriosis in RTE food a priority initiative with the aim to achieve the 50% reduction by 2005. Furthermore the federal government established a goal of working with industry and consumers to achieve an additional reduction in listeriosis of 50% by 2010 (DHS, 2000; CFSAN, 2001). In Europe the product group of fruits and vegetables eaten raw was included in a coordinated program for the food control in 2002 due to a recommendation (2002/66/EG)

of the EU-committee (Anonymous, 2002b). Such concepts are in correspondence with the definition for Food Safety Objects (FSO), which have the focus on the target after analysing the problem (ICMSF, 2002).

There are specific problems that can be assigned to each of this product type. The microbial load of foods depends in principle on their bacterial contamination, possibilities of decontamination and the properties of the products which allow the bacteria to survive, to grow or to die. In the following the product related problematic and the state of knowledge will be described.

### **Microbiological safety of sprouts**

The group of minimally processed fruit and vegetables include prepared fruit salads or fruit combinations, pre-washed salad items, grated vegetables and sprouts. Most of these products are generally eaten without further processing. A basic reflection of possible contamination permits an assessment of the efficacy of measures to ensure the safety. It has to point out, that inner tissues of fruits and vegetables are basically sterile (Lund, 1992). On their surfaces, fruits and vegetables carry naturally a non-pathogenic epiphytic microflora that includes bacteria, yeasts and moulds representing many genera. About two thirds of the spoilage of fruits and vegetables is caused by moulds (Snowdon, 1991). In this process the genera *Aspergillus*, *Botrytis*, *Rhizopus*, *Pectobacterium* (formerly *Erwinia*) and *Sclerotinia* are commonly involved (Lund et al., 2000; Tournas, 2005). The spoilage is associated with pectinolytic or cellulolytic activity which results in softening and weakening of the plant structures. These are important barriers to prevent growth in the products of the contaminated microbes. The majority of bacteria found on plant surfaces are usually gram-negative and belong to the *Pseudomonas* group or *Enterobacteriaceae* (Lund, 1992). Fruits and vegetables can become contaminated by pathogens from human or animal sources during growth, harvest, transportation, further processing and handling (Beuchat, 1996). This microbial contamination may have an impact on consumer health. In Table 1 and 2, reported outbreaks due to the consumption of ready-to-eat vegetables and fruits are compiled. Despite of the lower pH of fruits, bacteria are able to survive these conditions for a while.

**Table 1** Examples of outbreaks of foodborne diseases associated with raw fruits or fruit product

Pathogen	Year	Location	Fruit type or product	No. of cases	No. of deaths	Reference
<i>Cryptosporidium parvum</i>	1993	Maine, USA	Unpasteurised apple juice	>160	0	Millard et al., 1994
<i>Cryptosporidium parvum</i>	1996	New York, USA	Unpasteurised apple juice	>20	0	CDC, 1996
<i>Cyclospora cayetanensis</i>	1995	Florida, USA	Raspberries	87	0	Koumans et al., 1998
<i>Cyclospora cayetanensis</i>	1996	USA	Raspberries	>1400	0	Fleming et al., 1998
<i>Cyclospora cayetanensis</i>	1997	USA, Canada	Raspberries	>1000	0	CDC, 1998a
<i>Cyclospora cayetanensis</i>	1998	Canada	Raspberries	315	0	Herwaldt, 2000
<i>Cyclospora cayetanensis</i>	1999	Canada	Blackberries	104	0	Herwaldt, 2000
Calicivirus	1997	Canada	Frozen raspberries	>200	0	Gaulin et al., 1999
Calicivirus	1998	Finland	Frozen raspberries	>500	0	Ponka et al., 1999
Hepatitis A Virus	1990	Georgia, USA	Frozen raspberries	28	0	Niu et al., 1992
Hepatitis A Virus	1997	USA	Frozen strawberries	258	0	Hutin et al. 1999
<i>E. coli</i> O157:H7	1993	Oregon, USA	Melons	9	0	Del Rosario and Beuchat, 1995
<i>E. coli</i> O157:H7	1996	USA	Unpasteurised apple cider	14	0	CDC, 1997a
<i>E. coli</i> O157:H7	1996	Washington, USA	Unpasteurised apple cider	6	0	Farber et al., 2000
<i>E. coli</i> O157:H7	1996	USA, Kanada	Unpasteurised apple cider	70	1	Cody et al., 1999
<i>E. coli</i> O157:H7	1998	Canada	Unpasteurised apple cider	14	0	Tamblyn et al., 1999
<i>E. coli</i> O157:H7	1999	Oklahoma, USA	Unpasteurised apple cider	7	0	Farber et al., 2000
<i>Salmonella</i> Javiana	1991	USA	Melons	39	0	Blostein, 1993
<i>Salmonella</i> Hartford	1995	Florida, USA	Unpasteurised orange juice	69	0	Cook et al., 1998
<i>Salmonella</i> Saphra	1997	California, USA	Melons	24	0	Moehle-Boetani et al., 1999
<i>Salmonella</i> Oranienburg	1998	Canada	Melons	22	0	Deeks et al., 1998
<i>Salmonella</i> Muenchen	1999	USA, Canada	Orange juice	>300	1	CDC, 1999
<i>Salmonella</i> Enteritidis	2000	USA	Citrus juice	14	0	Butler, 2000
<i>Salmonella</i> Poona	2000	USA	Melons	unknown	unknown	FDA, 2001
<i>Shigella flexneri</i>	1998	UK	Fruit salad	136	0	O'Brien, 1998

**Table 2** Examples of outbreaks of foodborne diseases associated with raw lettuce or salad products

Pathogen	Year	Location	salad type or product	No. of cases	No. of deaths	Reference
<i>Campylobacter jejuni</i>	1984	USA	Salad	330	0	Allen, 1985
<i>Campylobacter jejuni</i>	1996	USA	Lettuce	14	0	CDC, 1998b
<i>Clostridium perfringens</i>	1993	Canada	Salad	48	0	Styliadis, 1993
<i>Cryptosporidium parvum</i>	1997	USA	Onions	54	0	CDC, 1998°
<i>Cyclospora cayetannesis</i>	1997	USA	Baby lettuce	> 91	0	Herwaldt and Beach, 1999
<i>Calicivirus</i>	1992	Canada	Salad	27	0	FDA, 2001
<i>E. coli</i>	1993	USA	Carrots	47	0	CDC, 1994
<i>E. coli</i> O157:H7	1995	USA	Lettuce (romaine)	21	0	CSPI, 2001
<i>E. coli</i> O157:H7	1995	USA	Iceberg lettuce	30	0	CSPI, 2001
<i>E. coli</i> O157:H7	1995	Canada	Iceberg lettuce	23	0	Preston et al., 1997
<i>E. coli</i> O157:H7	1995	USA	Lettuce	70	0	Ackers et al., 1998
<i>E. coli</i> O157:H7	1996	USA	Mesclun lettuce	49	0	Hillborn et al., 1999
<i>E. coli</i> O157:H7	1998	USA	Salad	2	0	Griffin and Tauxe, 2001
<i>L. monocytogenes</i>	1979	USA	Tomatoes, celery	20	5	Ho et al., 1986
<i>L. monocytogenes</i>	1981	Canada	Vegetable mix	41	17	Schlech et al., 1983
Salmonella Javiana	1993	USA	Tomatoes	174	0	Lund et al, 2000
Salmonella Baildon	1999	USA	Tomatoes	87	3	Cummings et al., 2001
Salmonella Typhimurium	2000	UK	Lettuce	361	0	Horby et al., 2003
Salmonella Newport	2001	UK, Scotland	Salad vegetables	60	not reported	Ward et al., 2002
Salmonella Braederup, Salmonella Javiana	2004	Canada	Roma tomoatoes	561	not reported	CDC, 2005
Shigella sonnei	1994	Norway, Sweden, UK	Iceberg salad	118	0	Kapperud et al., 1995



Within ready-to-eat foods of plant origin, differences exist between salads, fruits and sprouts with regard to the development of the native microbial load. The microbial load on salads and fruits is that present at the time of harvesting. Through processing (e.g. skinning, cutting, slicing etc.) the plant parts, dust as well as the bacterial load will be removed. Basically the flora from the field is of hygienic relevance for salads and fruits, a psychrotrophic flora (responsible for spoilage) may be added during processing (e.g. water). Botanically the term sprout describes the plant part developing on the hypocotyle from the seed leaves. Mung bean sprouts for example are botanically not sprouts, but consist only of hypocotyle and seed leaves.

During manufacturing of sprouts, however, the bacterial load will increase because the conditions under which seeds are sprouted (2–7 days of sprouting, temperatures of 20–40°C and optimum water activity (near 1.0) are ideal for bacterial proliferation. At the first day of sprouting the total bacterial counts increase by 2 to 3 log units and attain a maximum level after 2 days (Fu et al., 2001). The native flora on the seeds is therefore of hygienic relevance, during sprouting the mesophilic bacteria will grow, psychrotrophic ones may be added during washing. The potential growth of human pathogens such as salmonellae and *E. coli* O157:H7 in these microbial communities is of major concern as seed sprouts have been implicated in several outbreaks of foodborne diseases, mainly caused by salmonellae and *Escherichia coli* O157: H7 (Table 3). The largest outbreak involved more than 6000 persons in Japan, and was associated with the consumption of radish sprouts. Therefore, in sprout production, the assurance of the absence of pathogens on seeds can be regarded as the critical control point, as defined by the Codex Alimentarius Commission (Anonymous, 1993).

Many studies have been performed to decontaminate seeds using methods such as irradiation, UV light, pulsed electric or magnetic fields, high pressure, heat treatments as well as disinfectants (FDA, 2001). Seeds have been soaked, dipped, sprayed and fumigated with a wide range of chemical compounds. Especially chlorine has been extensively tested (Jacquette et. al., 1996; Beuchat et al., 2001; Holliday et. al, 2001; Montville and Schaffner, 2004), and further agents used in studies were gaseous acetic acid ( Delaquis et al., 1999), ammonia (Himathongkham et al., 2001) calcinated calcium (Bari et al., 2003) and electrolyzed oxidizing water (Kim et al., 2003). Among the methods mentioned above, washing is of major importance. In Table 4 the efficacy of washing agents to decontaminate the seed are compiled.

To minimize the risk of food poisoning, it has been recommended by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) to achieve a 5-log reduction of

**Table 3** Examples of outbreaks of foodborne diseases associated with raw seed sprouts

<b>Pathogen</b>	<b>Year</b>	<b>Location</b>	<b>Seed type</b>	<b>No. of cases</b>	<b>No. of deaths</b>	<b>Reference</b>
<i>Salmonella</i> Saint-Paul	1988	UK	mung beans	143	0	O'Mahony et al., 1990
<i>Salmonella</i> Gold Coast	1989	UK	Cress	31	0	NACMCF, 1999
<i>Salmonella</i> Bovismorbificans	1994	Sweden / Finland	Alfalfa	492	0	Ponka et al., 1995
<i>Salmonella</i> Montevideo	1996	USA	Alfalfa	>500	1	NACMCF, 1999
<i>E. coli</i> O157:H7	1996	Japan	Radish	>6000	2	Watanbe et al., 1999
<i>E. coli</i> O157:H7	1997	USA	Alfalfa	108	0	CDC, 1997b
<i>Salmonella</i> Senftenberg	1998	USA	Radish	60	0	NACMCF, 1999
<i>Salmonella</i> München	1999	USA	Alfalfa	157	0	Proctor et al., 2001
<i>E. coli</i> O157:H7	1998	Japan	alfalfa / clover	8	0	FDA, 2001
<i>Salmonella</i>	2000	USA	mung beans	45	0	FDA, 2001
<i>Salmonella</i> Enteritidis PT4b	2000	Netherlands	soy beans	25	0	FDA, 2001
<i>Salmonella</i>	2001	USA	mung beans	30	0	Honish, 2001
<i>Salmonella</i> Kottbus	2001	USA	Alfalfa	31	0	Winthrop et al., 2003

**Table 4** Efficiency of washing in reduction of bacterial counts on seeds

Challenge organism	Seed type	Treatment	Results of treatment	References
<i>Salmonella</i>	Alfalfa	1800 ppm Ca(OCl) <sub>2</sub> /2000 ppm NaOCl/ 6% H <sub>2</sub> O <sub>2</sub> /80% ethanol	up to 3 log units reduction	Beuchat, 1998
<i>Salmonella</i> Stanley	Alfalfa	Chlorine and hot water	up to 2 log units reduction	Jaquette et al., 1996
<i>E. coli</i> O157:H7	Radish	0.4% (wt/vol) calcinated calcium	3 log units reduction	Bari et al., 1999
<i>E. coli</i> O157:H7	Alfalfa	up to 2000ppm Ca(OCl) <sub>2</sub> / 500 ppm acidified ClO <sub>2</sub> /70% ethanol/8% H <sub>2</sub> O <sub>2</sub>	up to 3 log units reduction	Taormina and Beuchat, 1999
<i>S. Typhimurium</i> , <i>E. coli</i>	Alfalfa, Mung bean	180 or 300 mg/l of ammonia	2-3 log units reduction on alfalfa, 5-6 log units on mung beans	Himathongkham et al., 2001
<i>Salmonella</i> , <i>E. coli</i>	Alfalfa	20.000 ppm chlorine/8% H <sub>2</sub> O <sub>2</sub> /1% Ca(OH) <sub>2</sub> + 1% Tween80	1.6 to 3.9 log units reduction; overall Ca(OH) <sub>2</sub> most effective	Holliday et al., 2001
<i>Salmonella</i> , <i>E. coli</i> O157:H7	Mung bean	up to 3% (wt/vol) Ca(OCl) <sub>2</sub>	reduction up to 5 log units	Fett, 2002
<i>E. coli</i> O157:H7	Alfalfa	up to 21 ppm ozone and hot water	reduction up to 3.6 log units	Sharma et al., 2002
<i>Salmonella</i>	Alfalfa	Electrolyzed oxidizing water (84 ppm of active chlorine) for 10min	reduction of 1.5 log units	Kim et al., 2003
<i>L. monocytogenes</i>	Alfalfa	21.8 ppm aqueous ozone for 10 and 20 min.	no significantly reduction of <i>L. monocytogenes</i> numbers	Wade et al., 2003

pathogens on seeds used for sprout production (NACMCF, 1999). As it had been shown that treatment with 20,000 ppm calcium hypochlorite may be adequate (Beuchat et al., 2001; Fett, 2002), this type of treatment is in the US commonly used for seed disinfection. However, in certain countries such as Germany, the application of chlorine or other disinfectants for the production of organic food is not accepted. An alternative is a hot water treatment of the seeds. Such decontamination step can be seen as a first hurdle to ensure the food safety of sprouts.

A second hurdle may be the application of biological methods such as the use of antagonistic plant ingredients as well as the addition of protective cultures. These may have a sustainable effect to prevent the growth of organism during the production process.

In general, biopreservation consists of the following principle: 1) the use of organisms to control growth of spoilage flora as well as pathogenic species 2) the use of microbial antagonistic compounds to control microbial growth and negatively affect viability of pathogens 3) natural plant defence principle such as microbial attack-induced resistance. Finally, non-pathogenic microorganisms that can compete with pathogens for physical space and nutrients (Parish et al., 2003).

There are a few published reports on the use of biocontrol agent, such as protective cultures, to prevent growth of human pathogens on sprouts. In studies described by del Campo et al. (2001) and Palmai and Buchanan (2002), the efficiency of selected strains in model media have been tested. Enomoto (2004) studied the effect of Enterobacteria against *Pseudomonas fluorescens*. Up to now no isolates from sprouts or the sprouting environments were effective *in praxi* to prevent the growth of pathogens in the course of the sprouting process.

Numerous studies have been performed to improve the food safety of sprouts, which were described above. None led to satisfactory results. The combination of a hot water treatment of seeds and the use of protective cultures was regarded as a more efficient method. The topic of this study was to prove the hypotheses.

## **Microbiological safety of cold-smoked salmon**

Cold-smoked salmon belongs together with fish salads and other smoked fish products to the group of ready-to-eat seafood. Food of animal origin is in general subjected to a greater hygienic risk than food of plant origin, as it may contain bacteria that cause zoonotic diseases. The native microflora on water fish and shellfish is dominated by psychrotrophic or psychrophilic gram-negative bacteria belonging to the genera *Pseudomonas*, *Moraxella*,

*Acinetobacter*, *Shewanella* and *Flavobacteria* (Liston, 1990). Gram positive organisms such as *Bacillus*, *Micrococcus*, *Clostridium* and *Corynebacterium* have also been found in varying proportions. Lactic acid bacteria are seldom part of the dominant flora, but are present at levels of 10-1000 cfu/g (Rachman et al., 2004). The microbial flora of freshly harvested farmed fish is similar to the flora found on wild fish. However, as the microflora is a reflection of the environment, it is not surprising that aqua cultured fish are more likely to be contaminated with certain non-indigenous species, because of the closer proximity of fish farms to human or animal populations and their waste (Shewan, 1977). Huss et al. (1995a) have shown that *L. monocytogenes* is frequently isolated from fresh water and polluted seawater, but not from virtually unpolluted ocean or spring water.

*Listeria monocytogenes* is a short (0.5 µm in diameter by 1 to 2 µm long) gram positive, non-spore-forming rod. Infections of humans can result in listerioses, a disease whose symptoms include septicaemia, meningitis and abortion. Cells of *L. monocytogenes*, ingested with the food, cross the barrier of the intestinal tract and are then engulfed by white blood cells and transported to different parts of the body. The bacteria are able to grow and multiply within cells and spread directly from cell to cell. All strains of *L. monocytogenes* appear to be pathogenic but their virulence, as defined in animal studies, varies substantially (Farber, 2002). Listerioses is an opportunistic infection that most often affects those with severe underlying disease, pregnant women and the elderly. Important characteristics of this organism are its ability to grow at temperatures of 1-45°C, at pH-values of 4.3-9.5, at water activity of >0.90, and in salt concentrations higher than 10% (Farber et al., 1992; Tienunggoon et al., 2000)

The microflora of a fish product such as cold-smoked salmon reflects the indigenous flora itself, the microflora of the processing environment as well as the conditions during manufacturing and storage. The manufacturing steps of cold-smoked fish often reduce the number of microorganisms exert a strong influence on the composition of the microflora. Generally, *Pseudomonades* and *Shewanella putrefaciens* become predominant during aerobic storage conditions (Gram et al. 1987), whereas vacuum or CA-conditions favour the growth of lactic acid bacteria, psychrotrophic *Enterobacteriaceae* or marine vibrios (Gram and Huss, 2000). Among foodborne pathogens that may be present on cold-smoked salmon, *L. monocytogenes* is of special concern. Several studies have reported a prevalence of 6-36% in cold smoked salmon and other RTE fish products (Ben Embarek, 1994; Lyhs et al., 1998; Dominguez et al., 2001; Becker et al., 2002; Farber, 2002; Gombas et al., 2003; FDA, 2003; Nakamura et al., 2004).

The experience has shown that contamination mostly occurs after catching, mainly at handling on board, and later along the production chain. Colburn et al. (1990) found *Listeria* species in 81% of freshwater samples and 30% of estuarine cost water samples. Due to the studies of Eklund et al. (1995) and Rorvik (2000), initial and most important source of contamination in processing plants is the surface (skin, mucus, tail head) and the intestinal cavity of the fish at the time of decapitation, peeling and filleting (Dauphin et al., 2001; Hoffmann et al., 2003). In contrast to these findings, Bagge-Ravn et al. (2003), Gall et al. (2004), Lappi et al. (2004a) as well as Nakamura et al. (2006) showed in their studies that *Listeria monocytogenes* during production process appears to be a major source of finished product contamination. The strains persist at plants, proliferate in the environment during warmer seasons, and contaminate products during manufacturing processes (Vogel et al., 2001).

The manufacturing process of cold-smoked salmon includes the preservation steps: chilling (<5°C), salting (<6% [w/w] NaCl), drying (1-6h, 20-28°C), smoking (3-8h at max. 30°C) and packaging. The products usually have a shelf live of 2-4 weeks at 5°C. The fact, that *L. monocytogenes* has been often isolated in cold-smoked salmon at the manufacturing level as well as in vacuum-packaged products at the retail shows that the organism can survive the cold smoking process and may grow at temperature/NaCl regimes prevailing in the course of cold storage (Gyer and Jemmi, 1991; Jorgensen and Huss, 1998; Feldhusen et al., 2001; Lappi et al., 2004b).

There are several cases of listerioses reported worldwide, that have been linked to the consumption of ready-to-eat fish products (Table 5). The FAO/WHO expert consultation has considered smoked fish among the RTE foods of the highest risk to consumers (FAO/WHO, 2004). To protect the consumers, the FDA of the U.S. and certain European countries (e.g. France, Australia and Italy) imposed a “zero tolerance”, whereas others (e.g. Germany, the Netherlands, Sweden, Canada and Denmark) have set a limit of less than 100 cfu/g at the time of consumption. The reasons for these different originate from the variation of the Appropriate Level of Protection (ALOP) in each country, which is measured in terms of “probability of disease” or “numbers of cases per year” (ILSI, 2005).

To improve the safety of cold smoked salmon, preservatives such as sodium nitrate and sodium lactate have been used to reduce the levels of *Listeria monocytogenes* (Pelroy et al. 1994a; Pelroy et al. 1994b; Su and Morrissey 2003). However, the application of preservatives interfere either with the sensory properties and/or is not permitted. Eklund et al. (1995), Tompkin (2002) and Scott et al. (2005) concluded that efforts to control

*L. monocytogenes* in the food processing plant environment can reduce the frequency and the level of contamination, but it is not possible to completely eliminate the organism from the processing plant or to eliminate the potential for contamination of finished products totally with the current given technology.

An alternative process to the use of chemicals is the biopreservation with the aid of protective cultures. Protective cultures are preparations consisting of living microorganisms (plain cultures or culture concentrates), which are added to foods in order to reduce risks arising from the presence of pathogenic or toxinogenic microorganisms (Hammes, 2004). The efficacy of protective cultures rests on two basic principles: 1) competitive exclusion, e.g. competing for nutrients as well as better adaptation to the environment and 2) formation of antagonistic compounds, such as acids (protons and acid residues [acetate, propionate, lactate, formate], bacteriocins (ribosomally synthesized peptides, proteins and proteinaceous compounds (e.g. lanthionine), antibiotics and others, e.g. benzoic acid, H<sub>2</sub>O<sub>2</sub>, diacetyl, etc.

Lactic acid bacteria (LAB) are possible candidates for the use as protective cultures on smoked salmon, as they have been frequently isolated as dominant flora. (Paludan-Mueller et al., 1998; Truelstrup Hansen, 1998; Rachmann et al., 2004). Furthermore several LAB strains have the potential to produce a variety of inhibitory substances (Rodgers, 2001). In literature, there are several publications describing the use of LAB (Huss et al., 1995b; Duffes, 1999; Duffes et al., 1999a; Nilsson et al., 1999; Duffes et al., 2000; Yamazaki et al., 2003, Alves et al., 2005) and/ or their antagonistic products as antilisterial compounds (Szabo et Cahill, 1999; Duffes et al., 1999b; Katla et al., 2001; Nilsson et al., 2004; Ghalfi et al., 2006). Most effective against *Listeria monocytogenes* with acceptable sensorial properties was a *L. sakei* strain used on cold smoked salmon as well as in a fish juice model, with a reduction of *L. monocytogenes* by 2 log units as well as 6 log units, respectively (Nilsson et al. 2004). All these studies have been conducted with smoked salmon in a laboratory scale or with salmon juice as a model system.

Numerous studies have been performed to improve the food safety of cold smoked salmon, which were described above. None led to satisfactory results. The efficiency of protective cultures, shown under the conditions of practice in a smokehouse, can be regarded as an alternative proof of safety assurance. It was the aim of this study to prove the hypotheses.

**Table 5** Outbreaks of listerioses associated with fish and fishery products

Year	Location	No. of cases	No. of deaths	Food	Reference
1989	USA	2	0	Shrimp	FAO, 1999
1989	Italy	1	0	Fish	FAO, 1999
1991	Australia	2	0	Smoked mussels	Misrachi et et al, 1999
1992	New Zealand	4	0	Smoked mussels	Brett et al., 1998
1996	Canada	2	unknown	Crab meat	FAO, 1999
1994-1995	Sweden	8	2	Cold-smoked rainbow trout	Ericsson et al, 1997; Tham et al., 2000
1998	Finland	5	0	Cold-smoked rainbow trout	Miettinen et al., 1999



## **Aim of the study**

It was the aim of this study to improve the food safety of raw ready to eat food with special focus on sprouts and cold-smoked salmon using physical and biological preservation methods.

In the first part, hot water treatment of seeds had been investigated as an alternative decontamination procedure. As seeds of the various plants exhibit great variation with regard to heat sensitivity and requirements for optimum germination, it was essential to develop for industrial sprout production tailored processes that include especially time/temperature relationships and treatment sequences. As an additional hurdle, in prevention of the growth of pathogens, protective cultures have shown their efficiency in some food processes and adapted strains had to be found as tools efficient in this specific area. As a source of microorganism used as protective cultures, the indigenous bacterial association in soil had been studied.

In the second part, another group of protective cultures was studied. Bacteriocin producing strains of lactic acid bacteria had been investigated for their efficiency in reducing the numbers of listeria on cold-smoked salmon. The experiments had been performed under the conditions used in industrial scale of a smokehouse.

The study on sprouts was performed as a part of the research projects: 1) “Reduction of microbial contamination and extension of shelf-life of packaged ‘ready-to-use’ salads” (AiF-project No. 12817 N.) and 2) “Reduction of microbial contamination and extension of shelf-life of packaged ‘ready-to-eat’ salads by combination processes” (AiF-project No. 13931 N). The projects were guided under the supervision of Professors W. P. Hammes in collaboration with R. Carle.

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## Chapter 2

### Outline of the thesis

**Chapter 1** provides a general introduction of microbiological safety, common decontamination methods and the use of protective microorganism and the technology of manufacturing of sprouts and cold smoked salmon as examples for ready-to-eat foods.

**Chapter 3** describes the influence of different drying methods on the germination rate after heat treatment of mung bean seeds, defines time/temperature regimes to obtain viable seeds after heat treatment, and characterized inactivation of *Salmonella* Senftenberg W775 on mung bean seeds by determination of the D and z-values.

This Chapter has been published in Journal of Applied Botany:

Alexander Weiss and Walter P. Hammes. 2003. Thermal seed treatment to improve the food safety status of sprouts. Journal of Applied Botany. 77: 152-155

**Chapter 4** describes the effect of a hot water treatment of alfalfa, mung bean and radish seeds at various time/temperature regimes. For the inactivation of 3 strains of *S. Bovismorbificans*, *S. Senftenberg* W775 and *E. coli* O157:H<sup>-</sup> (isolate Bockemühl) respectively, D- and z-values were determined.

This Chapter has been published in European Food Research and Technology:

Weiss Alexander and Hammes, Walter P. 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H<sup>-</sup> on alfalfa, mung bean and radish seeds used for sprout production. Eur. Food Res. Tech. 211, 187-191

**Chapter 5** describes the characterization of the microbiota developing during germination of seeds to ready to eat sprouts. For mung bean and radish sprouts, the differences of the microbiota of seedling parts when grown hydroponically and in soil were studied by using the PCR-DGGE and bacterial culture with 16S rDNA sequence analyses. Dominating pseudomonas strains were isolated and investigated for their ability to compete with pathogens. Contributions to this work were provided by Diep Ha and Silke Grothe as part of their diploma thesis, which have been performed under my supervision. In the genetic part Christian Hertel acted as supervisor.

This Chapter has been submitted for publication in Systematic and Applied Microbiology.

Alexander Weiss, Christian Hertel, Silke Grothe, Diep Ha, and Walter P. Hammes. Characterization of the microbiota of sprouts and their potential for application as protective cultures.

**Chapter 6** describes the design of a process using *L. sakei* strains as protective culture to prevent the growth of *Listeria sp.* on cold-smoked salmon under the praxis conditions of a smokehouse.

This Chapter has been published in European Food Research and Technology:

Weiss Alexander and Hammes, Walter P. 2006. Lactic acid bacteria as protective cultures against *Listeria spp.* on cold-smoked salmon. Eur. Food Res. Tech. 222, 343-346

### **Coauthorships**

This work was supervised by Professor Dr. Walter P. Hammes. Part of chapter 5 was supervised by Priv. Doz. Dr. Christian Hertel.

## Chapter 3

### Thermal seed treatment to improve the food safety status of sprouts

#### Abstract

The use of chemicals to reduce microbial contaminations on raw materials for the production of organic food such as sprout seeds is not allowed in Germany. To develop an alternative decontamination procedure, we studied the effect of hot water at various time/temperature regimes. Mung bean seeds were inoculated with  $>10^8$  cfu/g *Salmonella* Senftenberg W775 by immersion. This strain is known for its unusual high heat resistance. The seeds were dried and stored at 2 °C. The salmonella counts on the dried seeds remained unchanged during storage for 8 weeks. The contaminated seeds were treated at 55, 58 and 60 °C for 0.5-16 min. D-values of 3.9, 1.9 and 0.6 min, respectively, were determined and a z-value of 6.2 ( $r^2 = 0.99$ ) was calculated for the inactivation of *S. Senftenberg* W775 on the mung bean seeds. The thermal treatment at time/temperature regimes of 55°C/20 min, 60°C/10 min, 70°C/5 min and 80°C/2 min reduced the pathogens on the mung bean seeds by  $>5$  log units without affecting the germination rate of the seeds.

Alexander Weiss and Walter P. Hammes. 2003. Thermal seed treatment to improve the food safety status of sprouts. *Journal of Applied Botany*. 77: 152-155

## Introduction

The consumption of seed sprouts originates from far east countries and has spread in the past decades to other parts of the world, including Europe and the United States. Sprouts contain protein, carbohydrates, minerals and vitamins and are deemed to have a high nutritional value (Feng, 1997; Dohmen, 1987). On the German market an extraordinary great variety of different types of sprouts can be found including those from the following seeds: adzuki bean (*Phaseolus angularis*), alfalfa (*Medicago sativa*), broccoli (*Brassica oleracea convar. botrytis*), buckwheat (*Fagopyrum esculentum*), chickpea (*Cicer arietinum* L.), cress (*Lepidium sativum*), lentil (*Lens culinaris*), flax (*Linum usitatissimum*), mung bean (*Phaseolus aureus*), mustard (*Sinapis alba*), green and yellow pea (*Pisum sativum*), onion (*Allium cepa*), quinoa (*Chenopodium quinoa*), radish (*Raphanus sativus*), rice (*Oryza sativa* L.), rye (*Secale cereale*), sesame (*Sesamum indicum*), sunflower (*Helianthus annuus*) and wheat (*Triticum aestivum*). These products are consumed either as sprouts from a single type of seed or in mixtures of different types, usually as components of salads, sandwiches or, slightly cooked, as additions to soups. The consumption of seed sprouts has a 'healthy' image, but seed sprouts have been implicated in several outbreaks of foodborne diseases caused mainly by salmonella and *Escherichia coli* O157: H7 (Winthrop et al., 2003; Stewart, 2001a; Stewart, 2001b). The largest outbreak involved more than 6000 persons in Japan, and was associated with the consumption of radish sprouts (Watanbe, 1999). Potential sources of food borne pathogens include contaminated seeds, irrigation water and poor hygiene of food handlers. Under the conditions of good manufacturing practice (GMP), seeds are the primary source of these bacterial contaminants. Seeds may contain high microbial loads, commonly ranging between  $10^3$  to  $10^6$  cfu/g which are constituted mainly of pseudomonades, coliforms and lactic acid bacteria (Prokopowich and Blank, 1991; Splittstoesser et al., 1983; Robertson et al., 2002). The seeds are obtained from plants grown on open fields as any other crop seed, without special measures taking into account that they shall be used for production of sprouts serving as food. Bacterial growth is favoured by the sprouting conditions which are characterised by sprouting times of 2-7 days, temperatures of 20-40°C and optimum water activity for microbial growth (Taormina et al., 1999). At the first day of sprouting the total bacterial counts increase by 2 to 3 log units and attain a maximum level after 2 days (Fu et al., 2001). The potential growth of human pathogens such as salmonella and *E. coli* O157:H7 in these microbial communities is of major concern. Thus the assurance of the absence of pathogens on seeds can be defined as the critical control point (Codex Alimentarius Commission, 1993) in sprout production. Consequently, the National Advisory Committee

on Microbiological Criteria for Foods (NACMCF, 1999) recommended a 5 log units reduction of pathogens on seeds as a means of safety control in the production process, and it was shown that a treatment with 20,000 ppm calcium hypochlorite achieves this level of decontamination (Beuchat et al., 2001; Fett, 2002). The application of chlorine or other disinfectants for the production of organic food is not allowed in countries such as Germany. Therefore, physical or biological alternative treatments have to be developed to improve the safety of these ready to eat products. Seeds have already been treated with heat in combination with chemicals such as chlorine or ozonated water (Jaquette et al., 1996; Sharma et al., 2002; Scouten and Beuchat, 2002; Suslov et al., 2002). These studies were performed with alfalfa seeds, and it was observed that the load of pathogens did not yield a reduction by 5 logs without affecting the germination. To design an alternative decontamination without the use of chemicals, we studied the effect of hot water at various time/temperature regimes as the sole process step. We used mung bean seeds as raw material because these have the greatest market share in Germany.

## **Materials and Methods**

### **Microorganisms and culture conditions**

*Salmonella* Senftenberg W775 was obtained from Dr. Reisbrot (Robert Koch Institut, Werningerode). This strain was used as a worst case model because of its high heat resistance (Ng et al., 1969). *S. Senftenberg* was grown in S1 broth containing the following components per liter: 15 g tryptone, 3 g yeast extract, 6 g NaCl and 1 g glucose. The pH was adjusted to 7.5. To prepare inocula, *S. Senftenberg* was grown overnight in 1 l S1 broth in a rotary shaker (200 rpm, 37 °C). The cells were harvested by centrifugation and washed with sterile water. The pellet was suspended in 10 ml sterile water. To determine the salmonella counts, plant material homogenates in peptone solution were surface plated on bismuth sulphite agar (Merck) and incubated at 37 °C for 48 h.

### **Inoculation of the seeds**

The inoculum culture was added to 1 kg of mung bean seeds and mixed for 1min. Two methods were employed to obtain dry bacteria on the seeds: 1) Inoculated seeds were spread on petri dishes and dried within 24 h in a glass desiccator. 2) Seeds were spread on a metal grid and gently dried in a stream of air for 10 min. The temperature of the seeds was kept at 22-24 °C. The seeds were then stored 4 weeks in glass bottles at 2 °C. Under this conditions

the salmonella counts on the dried seeds remained unchanged at a level of  $1-5 \times 10^8$  cfu/g during storage for 10 weeks.

### **Thermal treatment of the seeds**

In an initial set of experiments the refrigerated seeds were allowed to equilibrate to ambient temperature (23 °C). To calculate D- and z-values for inactivation of *S. Senftenberg* W775 on the seeds, the treatments were conducted at 55, 58 and 60 °C for 2-20 min. To investigate, whether or not even higher heating temperatures can be applied to reduce the salmonella load by more than 5 log units without affecting the germination, contaminated seeds were also exposed to 70 °C (5 min) and 80 °C (2 min). For each experiment 5 g of inoculated seeds were added to sterile tap water (250 ml) and kept at the desired temperature with occasional agitation. During the thermal treatment the seeds were contained in a metal grid device placed in a beaker. Thereafter the seeds were cooled by immediate transfer into 45 ml peptone saline.

### **Calculation of D- and z-values**

At each sampling point, the salmonella counts were determined. Each experiment was repeated at least three times. For each temperature, the linear regression line was determined. The decimal reduction times ( D-values, in min) were obtained by the formula:  $D = t/(\log A - \log B)$ , where t equals heating time in min, A the initial number of *S. Senftenberg* on the seeds, and B the final number of *S. Senftenberg* on the seeds. The z- value (°C) is the negative inverse of the slope of the linear regression line for the log D-values.

### **Microbiological analyses**

To determine the initial counts of *Salmonella* Senftenberg W775 on contaminated seeds, 10 g were placed in 90 ml sterile peptone saline water in a flask. Seeds were homogenized by an Ultra-Turrax T25 (1 min, 20500 rpm/ min). The suspension was serially diluted in sterile peptone saline water and surface plated (0.1 ml) in duplicate.

### **Determination of seed germination rates**

Heat treated mung bean seeds were tested for their viability, i.e. the percentage capable to germinate. 5 g of the seeds were placed on a moistened filter paper in sterile petri dishes. Water was added after 24 h to provide sufficient moisture. After 48 h at 35 °C seeds were visually examined and the percentage of germination was calculated. Untreated sample served as control.

## Results

### Different drying methods affecting the germination

Experiments were performed with the aim to reduce the salmonella counts on the seeds by more than 5 logs. The initial load was adjusted to  $>10^8$  CFU/g of salmonella and to simulate the worst case situation and to operate close to practical conditions, the bacteria on the seeds were dried. Based on the experience that hydrated bacteria are more sensitive to heat than dried organisms, we initially pre-soaked the seeds in water before heat treatment. It was observed that this process manipulation, consisting of addition of the salmonella culture to the plant seeds and drying for 24 h (method 1), was sensed by the plant seeds and resulted in reduction of the germination rate (Table 1). With drying method 2 the germination rate of inoculated, thermally treated seeds was not affected. Therefore, the further experiments were carried out according to method 2.

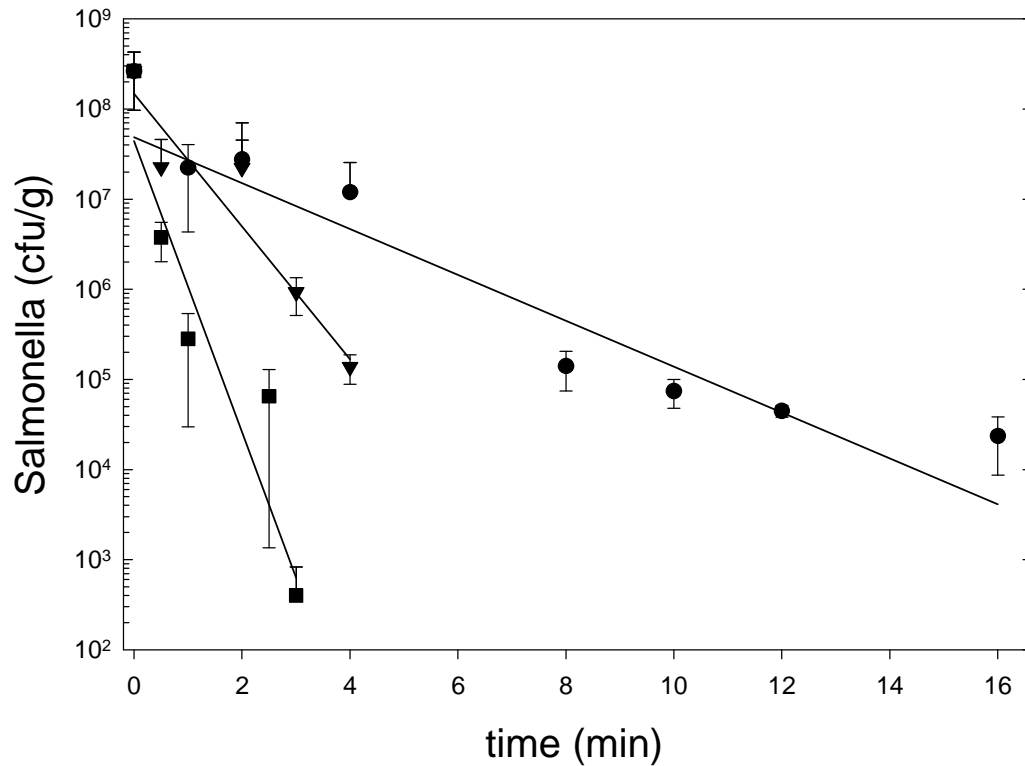
**Tab. 1:** Germination rates of variably dried seeds after thermal treatment

Pre-soaking time (min)	Treatment		Germination rate (%)			
	Temperature (°C)	Time (min)	Treated seeds (method 1)	Non treated seeds (method 1)	Treated seeds (method 2)	Non treated seeds (method 2)
10	70	7	55		99	
10	25	7		99		100
30	70	7	61		99	
30	25	7		100		99
30	80	2	71		100	
30	25	2		99		99

### Hot water treatment

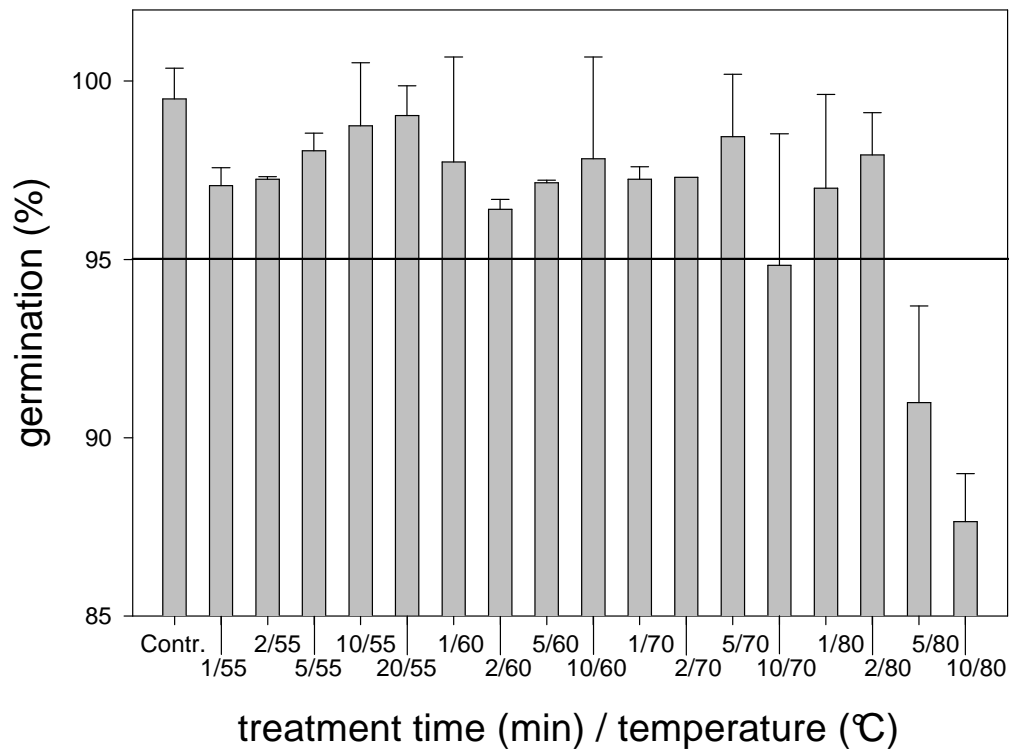
Figure 1 shows the thermal death time curves for *Salmonella* Senftenberg W775 at 55, 58 and 60 °C. A 3 log reduction of *S. Senftenberg* was achieved at 55 °C after 12 min, at 58 °C after 4 min and at 60 °C after less than 2 min. For each temperature the linear regression was determined and D- values of 3.9 min, 1.9 min and 0.6 min, respectively, were calculated.



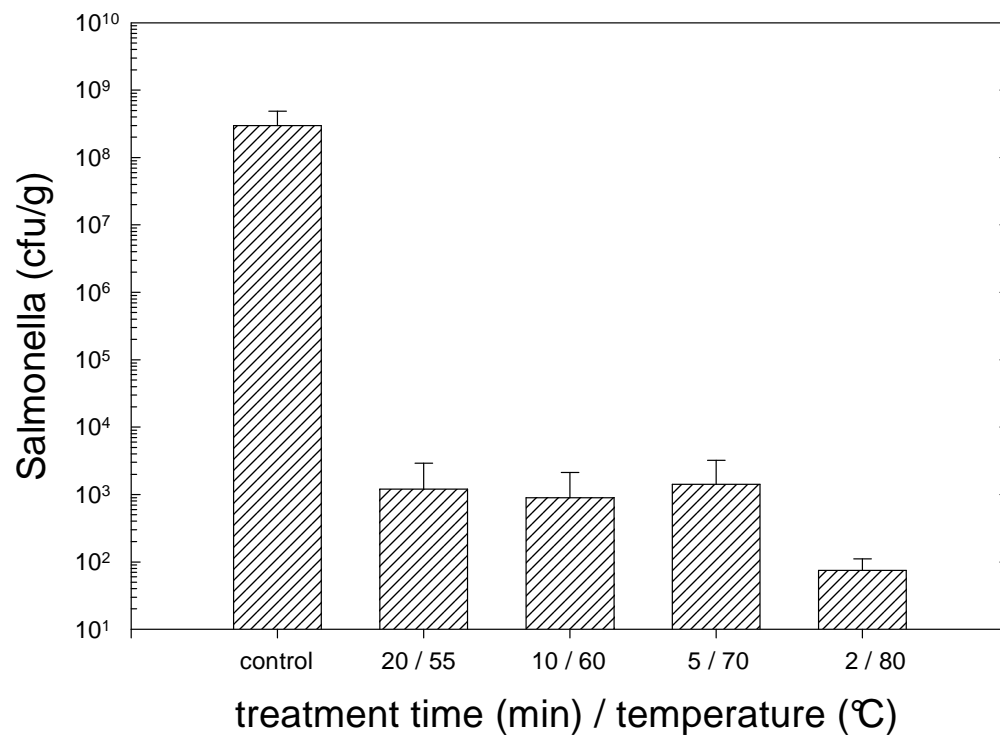


**Fig. 1:** Thermal death time curves for *Salmonella* Senftenberg W775, determined at 55 °C (●), 58 °C (▼) and 60 °C (■).

These values were used to calculate a  $z$ -value of 6.2 °C ( $r^2 = 0.99$ ) for *S. Senftenberg* W775 on mung bean seeds. The effect of different time/temperature regimes on the germination rates of mung bean seeds was studied at 55, 60, 70 and 80 °C. In practice, a germination rate >95 % is acceptable and this limit is marked in the compilation of the resulting values shown in Figure 2. Within the various regimes tested, values of 10 min at 70°C and 5 min at 80°C caused a reduction of seed germination below 95%. At the acceptable extreme time/temperature regimes the seeds were treated to investigate the decrease of *Salmonella* Senftenberg W775. The salmonella load decreased by >5 log units as depicted in Figure 3, at 80°C for 2 min the salmonella counts on the seeds were reduced even by >6 log units.



**Fig. 2:** The effect of different time/temperature regimes on germination rates of mung bean seeds.



**Fig. 3:** Reduction of *Salmonella* Senftenberg W775 on mung bean seeds after treatment within different time/temperature regimes

## Discussion

The basis for the recommendation to incorporate a 5 log reduction step in the production process for sprouts is a calculation performed by the NACMCF (1999). Quantitative analyses performed on seeds associated with disease upon consumption of sprouts revealed that the numbers of pathogens ranged between 1 and 6/100 g of seeds. Therefore, the worst case scenario for seed contamination was assumed to be 1 pathogen/10 g of seeds. It was also assumed that 50 kg of seeds is the amount of starting material for each batch of sprouts. This yields to 5000 pathogens per batch of sprouts. Thus, a 5 log treatment will yield 0.05 pathogens/batch. Taking into account that the bacterial counts increase rapidly during the first day of sprouting, it is reasonable to follow this recommendation. In the US it was found that a concentration of 20,000 mg/kg of calcium hypochlorite achieves this level of decontamination and this treatment became a recommended measure. As the use of disinfectants for the production of organic food is poorly accepted, its application like that of any chemical is an undesired step. Clearly the alternative is either not producing sprouts or to have indeed a rigorous concept that bases on the 5 log reduction step. Thermal treatment is a common method in food industry to achieve numerous effects, including the decontamination of food. However, seeds and bacteria contain living cells which are heat sensitive. These sensitivities differ however and it is known that seeds in dry status generally tolerate great stresses and, therefore, it can be assumed that heat regimes can be defined which are effective in killing bacteria without affecting the germination of the seeds. For mung beans we determined a z-value of 6.2 °C, which value is in the order of those known for endospores of *Bacillaceae* (>5.5 °C). Thus, the *Salmonella* Senftenberg W775 dried on the seeds simulated indeed a worst case situation. In our work we defined a wide range of time/temperature regimes for mung bean treatment achieving a 5 log reduction that can be used without affecting the germination rate (i.e. >95 %). A regime of 80 °C for 2 min reduced the load of salmonella even by more than 6 log units. We are aware that other types of seeds might be more sensitive to heat than mung beans. Therefore, a generalisation is not possible and the thermal treatment needs to be adapted specifically for each seed species. Thermal processes are common in food industry and their adaptation even by small sprout producers should not be a main obstacle.

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## Chapter 4

### **Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H<sup>-</sup> on alfalfa, mung bean and radish seeds used for sprout production**

#### **Abstract**

We studied the effect of hot-water treatment at various time/temperature regimes to design a decontamination process which is consistent with the recommendation of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) to reduce pathogens on seeds by 5log cfu/g. Alfalfa, mung bean and radish seeds were inoculated by immersion with more than  $10^7$  cfu/g of enterobacteria (*Salmonella* Senftenberg W775, *S. Bovismorbificans* and *Escherichia coli* O157:H<sup>-</sup>), dried and stored at 2 °C. The numbers of salmonellae and *E. coli* O157:H<sup>-</sup> on these seeds remained unchanged during storage for 8 weeks. To achieve sprouting rates of more than 95%, time-temperature regimes were defined. The thermal treatment of contaminated mung bean (2–20 min for 55–80 °C), radish and alfalfa seeds 0.5–8 min (53–64 °C) reduced all pathogens by more than 5log cfu/g. For *S. Senftenberg* W775 on radish seeds, *D* values of 3.2, 1.9 and 0.6 min were determined for exposure at 53, 55 and 58 °C and a *z* value of 6.2 °C was calculated. For alfalfa seeds, the respective *D* values were 3.0, 1.6, and 0.4 min and the *z* value was the same as that determined for radish seeds.

Weiss Alexander and Hammes, Walter P. 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H<sup>-</sup> on alfalfa, mung bean and radish seeds used for sprout production. Eur. Food Res. Tech. 211, 187-191

## Introduction

The use of seed sprouts as food originates from Far East countries and has spread in the past decades to parts of the Western world. These products are consumed as sprouts of a single type of seed or as mixtures of different types. They are usually eaten raw as components of salads, or slightly cooked in various dishes. We have found on the German market an extraordinary great variety of 24 different types of sprouts, namely, adzuki bean (*Phaseolus angularis*), alfalfa (*Medicago sativa*), beetroot (*Beta vulgaris* L. ssp. *vulgaris* var. *conditiva* Alef.), broccoli (*Brassica oleracea* convar. *botrytis*), buckwheat (*Fagopyrum esculentum*), chickpea (*Cicer arietinum* L.), cress (*Lepidium sativum*), lentil (*Lens culinaris*), flax (*Linum usitatissimum*), mung bean (*Phaseolus aureus*), mustard (*Sinapis alba*), green and yellow pea (*Pisum sativum*), onion (*Allium cepa*), quinoa (*Chenopodium quinoa*), radish (*Raphanus sativus*), red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*), rice (*Oryza sativa* L.), rye (*Secale cereale*), sesame (*Sesamum indicum*), soy (*Glycine max* (L.) Merr.), spelt (*Triticum spelta*), sunflower (*Helianthus annuus*) and wheat (*Triticum aestivum*). The most popular are alfalfa, mung beans and radish. Seed sprouts have a “healthy” image, as they contain protein, carbohydrates, minerals and vitamins and are deemed to have a high nutritional value [1, 2]. On the other hand, sprouts have been involved in numerous outbreaks of food-borne diseases [2–7]. Most of them were traced back to seeds contaminated with salmonellae and *Escherichia coli* O157:H7<sup>−</sup>, followed by *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Aeromonas hydrophila* [5, 7–9]. The largest outbreak was associated with the consumption of radish sprouts involving more than 6,000 people in Japan [6]. Seeds may be contaminated by high microbial loads, commonly ranging between 10<sup>3</sup> and 10<sup>6</sup> cfu/g, which include mainly pseudomonads, coliforms and lactic acid bacteria [10–14]. The seeds used for sprouting are obtained from plants grown on open fields as any other crop seed, without special measures, and the common sprouting conditions (2–7 days of sprouting, temperatures of 20–40 °C and optimum water activity) favor bacterial growth [4, 15]. Therefore, in sprout production, the assurance of the absence of pathogens on seeds can be regarded as the critical control point, as defined by the Codex Alimentarius Commission [16]. Many studies have been performed to decontaminate seeds using methods such as irradiation, UV light, pulsed electric or magnetic fields, high pressure, heat treatments as well as disinfectants [5]. Seeds have been soaked, dipped, sprayed and fumigated with a wide range of chemical compounds. Especially chlorine has been extensively tested [17–21] and further agents used in studies were gaseous acetic acid [22] ammonia [23], calcinated calcium [24] and electrolyzed oxidizing water [25]. It has been recommended by the National Advisory



Committee on Microbiological Criteria for Foods (NACMCF) to achieve a 5-log reduction of pathogens on seeds used for sprout production [3] and it had been shown that treatment with 20,000 ppm calcium hypochlorite is adequate [17, 26]. It has, however, been observed that this method may not always be sufficiently effective in reducing the numbers of pathogens from laboratory-inoculated seeds [20, 21].

The application of chlorine or other disinfectants for the production of organic food is not accepted in certain countries such as Germany. Therefore, physical or biological alternative treatments have to be developed to improve the safety of these ready-to-eat products. Mung bean seeds have already been treated with hot water and a 5-log reduction of *Salmonella* Senftenberg W775 was achieved without affecting the germination [27]. To design an alternative decontamination without the use of chemicals for additional seeds, we studied the effect of hot water at various time/temperature regimes as the sole decontamination step.

## Materials and Methods

### Microorganisms and culture conditions

*S. Senftenberg* W775 (LTH 5703) and three strains of *S. Bovismorbificans* (LTH 5704, 5705, 5706) were obtained from the National Reference Centre for Salmonellae and Other Enterics (Robert Koch Institute, Werningerode, Germany). A clinical isolate of *E. coli* O157:H<sup>-</sup>;SLT<sup>-</sup> (LTH 5807), was obtained from Professor Bockemühl (Institute for Hygiene and Environment Hamburg, Germany). *S. Senftenberg* W775 was used as a worst-case model because of its high heat resistance [28]; the *S. Bovismorbificans* strains were isolates obtained from sprouts. The salmonellae and the *E. coli* were grown in standard count broth (Merck, Darmstadt). To prepare inocula, bacteria were grown overnight in 1 l standard count broth in a rotary shaker (200 rpm, 37 °C). The cells were harvested by centrifugation and washed with sterile water. The pellet was suspended in 10 ml sterile water and served as inoculum.

### Inoculation of seeds

Three batches of seeds were prepared by inoculation with *S. Senftenberg* W775, *E. coli* O157:H<sup>-</sup> and a mix of the three *S. Bovismorbificans* strains, respectively. The inocula were added to 1 kg of mung bean seeds and 0.5 kg of radish or alfalfa seeds and mixed for 1 min. The contaminated seeds were spread on a metal grid, gently dried at 22–24 °C in a stream of air for 10 min and stored for 4 weeks in glass bottles at 2 °C. Under these conditions, the numbers of salmonellae and *E. coli* remained unchanged on the dried seeds at a level of more than 10<sup>7</sup> cfu/g during storage for 10 weeks.

### Thermal treatment of the seeds

For each experiment refrigerated, inoculated seeds (5 g) were adjusted to ambient temperature (23 °C), added to sterile tap water (250 ml) and kept at the desired temperature with occasional agitation. During the thermal treatment, the seeds were contained in a metal grid device placed in a beaker and the temperature was continuously controlled. The seeds were then cooled by immediate transfer into 45 ml peptone saline.

To calculate  $D$  and  $z$  values for inactivation of *S. Senftenberg* W775 on radish and alfalfa seeds, the treatments were conducted at 53, 55 and 58 °C for 1–8 min. At each sampling point, the *S. Senftenberg* W775 counts were determined. Each experiment was repeated at least three times. For each temperature, the linear regression was determined. The decimal reduction times ( $D$  values, in minutes) were obtained by the formula  $D=t/(\log A-\log B)$ , where  $t$  is the heating time in minutes,  $A$  is the initial number of *S. Senftenberg* on the seeds, and  $B$  is the final number of *S. Senftenberg* on the seeds. The  $z$  value (degrees Celsius) is the negative inverse of the slope of the linear regression for the  $\log D$  values. To investigate whether or not even higher heating temperatures can be applied to reduce the load of pathogens by more than 5 log units without affecting the germination, contaminated seeds were also exposed to 55–80 °C for 2–20 min (mung bean), 55–62 °C for 2–8 min (radish) and 55–62 °C for 2–10 min (alfalfa).

### Microbiological analyses

To determine the microbial counts of the contaminants, seeds or sprouts (10 g) were transferred into 90 ml sterile peptone saline and were homogenized with the aid of an Ultra-Turrax T25 (1 min, 20,500 rpm). The suspension was serially diluted in sterile peptone saline and surface-plated in duplicates. *Salmonellae* and *E. coli* LTH5807 were cultured on bismuth sulfate agar (Merck, Darmstadt) and Fluorocult *E. coli* O157 agar (Merck, Darmstadt) at 37 °C for 48 h, respectively.

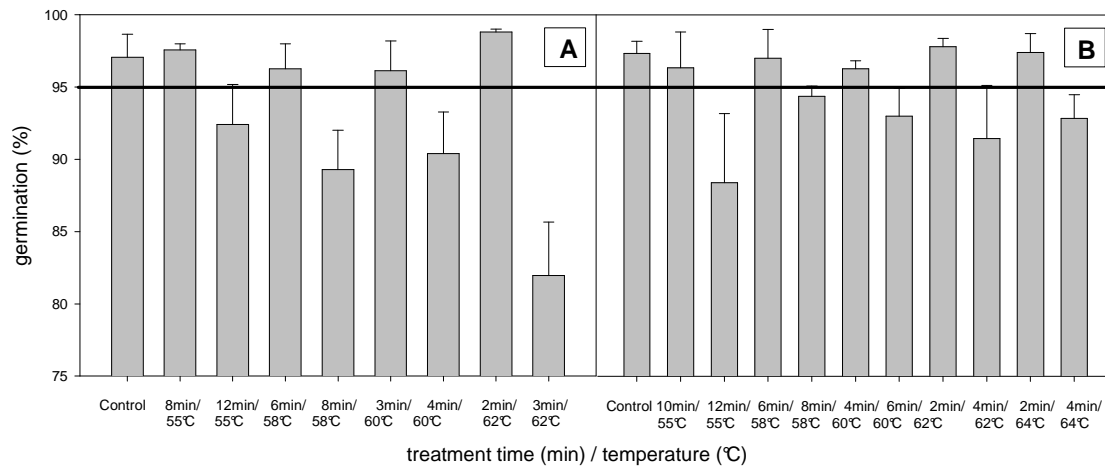
### Determination of seed germination rates

Heat-treated seeds were tested for their ratio of germination. For that purpose, 10 g of mung bean seeds and 5 g of radish and alfalfa seeds, respectively, were placed on a moistened filter paper in sterile petri dishes. Water was added after 24 h to maintain saturating conditions. After 48 h at 30–35 °C, the seeds were visually examined and the percentage of germination was calculated. An untreated sample served as a control.

## Results

### Germination of radish and alfalfa seeds

The germination ratio resulting from exposure to various time/temperature regimes was determined for alfalfa and radish seeds. As shown in Fig. 1, hot-water treatment of radish seeds can be performed in a range of 55–62 °C for 2–8 min (Fig. 1a), whereas alfalfa seeds permit a treatment range of 55–64 °C for 2–10 min (Fig. 1b). These conditions differ from those determined for mung bean seeds [27], which could be treated from 55 to 80 °C for 2–20 min. The application of higher temperature reduced clearly the sprouting rate. According to the experience of sprouters, in practice, a germination rate of more than 95% is acceptable and this limit is marked in the compilation of the resulting values shown in Fig. 1.

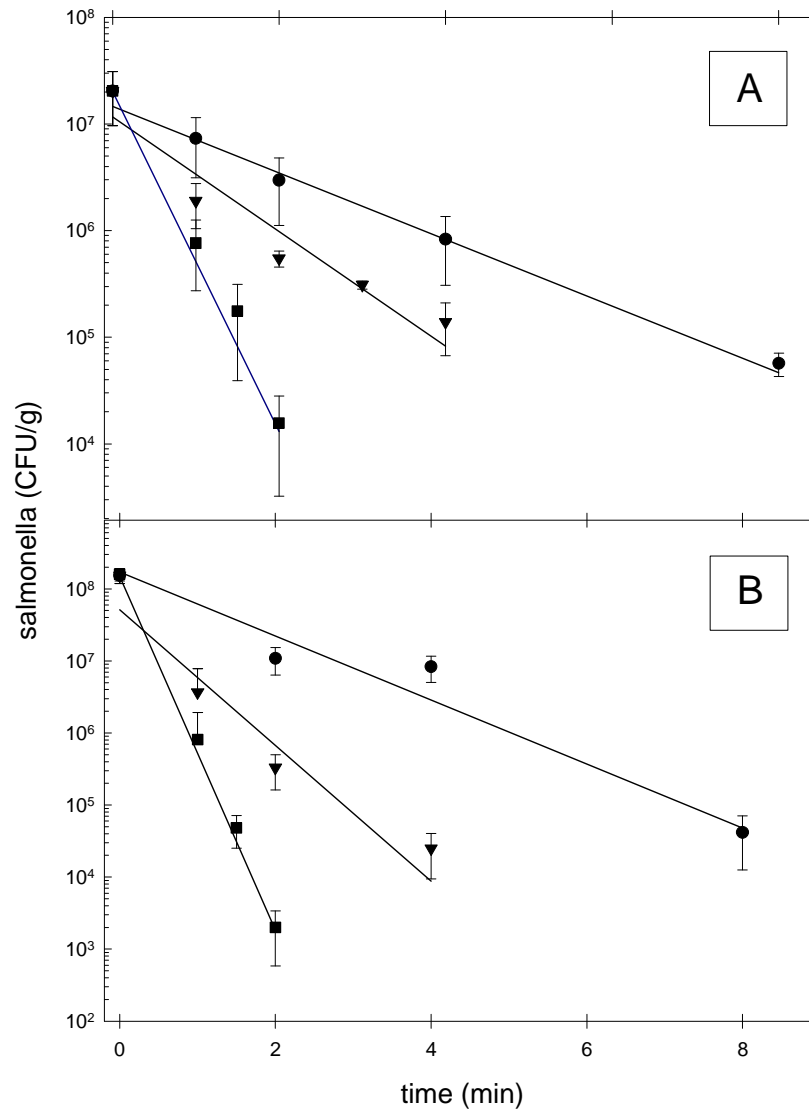


**Fig. 1** The effect of time/temperature regimes on germination of radish (A) and alfalfa (B) seeds. The line indicates the 95% germination ratio.

### Determination of D-and z-values

As shown in Fig. 2a, the thermal death time curves for *S. Senftenberg* W775 on radish seeds, a 3-log reduction of *S. Senftenberg* was achieved at 58 and 55 °C after 2 and 4 min, and at 53 °C a 2.5-log reduction was measured after 8 min. From the linear regression *D* values of 3.2, 1.9 and 0.6 min, respectively, were calculated. These values were used to calculate a *z* value of 6.2 °C ( $r^2=0.98$ ) for *S. Senftenberg* W775 on radish seeds. Figure 2b shows the thermal death time curves for *S. Senftenberg* W775 on alfalfa seeds. A greater than 3-log reduction of *S. Senftenberg* was achieved at 58 °C after 1.5 min, at 55 °C after 4 min and at 53 °C after 8 min, respectively. The *D* values determined were 3.0, 1.6 and 0.4 min,

respectively. These values allowed us to calculate a  $z$  value ( $r^2=0.99$ ) for *S. Senftenberg* W775 on alfalfa seeds which was identical with that for radish seeds.



**Fig. 2** Thermal death time curves for *Salmonella* Senftenberg W775 on radish (A) and alfalfa seeds (B) determined at 53°C (●), 55°C (▼) and 58°C (■).

### Hot water treatment for a 5-log reduction of pathogen

The effect of different time/temperature regimes on the reduction of pathogens on seeds is compared in Table 1. All chosen regimes resulted in germination rates of more than 95%. On alfalfa seeds, the counts of *S. Senftenberg* W775 decreased by more than 5log cfu/g, whereas on radish seeds temperatures ranging from 58 to 62 °C caused a reduction by more than 7log

cfu/g. The strain mix of *S. Bovismorbificans* was reduced on mung bean seeds by more than 5log cfu/g and on alfalfa seeds by more than 6log cfu/g. The treatment of radish seeds at 55 and 58 °C decreased the counts of *S. Bovismorbificans* by more than 5log cfu/g, whereas treatments at higher temperatures (and a shorter exposure time) did not achieve this limit. Temperatures of 60 °C or higher for mung bean seeds as well as 58 °C or higher for radish seeds, even brought about a greater than 7-log reduction of *E. coli* O157:H<sup>-</sup>. To reach the more than 5-log reduction with this strain on alfalfa seeds, temperatures of 58 °C or higher were required. At 55 °C, the reduction of *E. coli* O157:H<sup>-</sup> on all kinds of seeds was less than 5log cfu/g.

**Table 1** The effect of different time temperature regimes on the reduction of pathogen counts on mung bean, radish and alfalfa seeds.

Seed type	Treatment		Reduction (log CFU/g)		
	Temperature (°C)	Time (min)	<i>Salmonella</i> SenftenbergW775	<i>Salmonella</i> Bovismorbificans	<i>Escherichia coli</i> O157:H <sup>-</sup>
Mung bean	55	20	- <sup>a</sup>	5.22 ± 0.43	4.31 ± 0.31
	60	10	- <sup>a</sup>	5.40 ± 0.65	>7.16
	70	5	- <sup>a</sup>	5.25 ± 0.65	>7.16
	80	2	- <sup>a</sup>	6.55 ± 0.07	>7.16
Radish	55	8	5.40 ± 0.48	5.45 ± 0.31	3.83 ± 0.03
	58	6	>7.15	6.03 ± 0.18	>7.15
	60	3	>7.15	4.26 ± 0.37	>7.15
	62	2	>7.15	3.68 ± 0.27	>7.15
Alfalfa	55	10	>5.73	>6.00	4.44 ± 0.11
	58	6	>5.73	>6.00	5.15 ± 1.28
	60	4	>5.73	>6.00	5.91 ± 0.84
	62	2	>5.73	>6.00	5.06 ± 0.75

<sup>a</sup> determined previously by Weiss and Hammes [27]

## Discussion

In our studies we could show that hot-water treatment is an alternative to chemicals used to reduce the numbers of pathogens on seeds. It was observed that a 5-log reduction is achievable for salmonellae and *E. coli* O157 H<sup>-</sup> on alfalfa, mung bean and radish seeds with hot-water treatment as the sole decontamination step. Furthermore, we provided data on the efficacy of reducing *S. Senftenberg* W775 on radish and alfalfa seeds. The calculated *D* and *z* values are comparable with those known for endospores of *Bacillaceae* ( $z > 5.5$  °C) and are close to the values determined for mung bean seeds [27]. It is known that seeds in a dry status can tolerate stress, permitting their exposure to defined heat regimes that kill bacteria without affecting seed germination. We used *S. Senftenberg* W775 as worst-case model, and it is remarkable that it was possible to achieve on dried seeds a reduction of more than 5-log and to keep the germination rate above 95%. Similar results were obtained for *S. Bovismorbificans* on mung bean and alfalfa seeds. However, for *S. Bovismorbificans* on radish seeds, treatment for more than 6 min was necessary to follow the recommendation of the NACMCF, whereas exposure for 3 and 2 min at 60 and 62 °C, respectively, did not suffice.

Decontamination of seeds by hot-water treatment in combination with chemicals such as chlorine or ozonated water has already been investigated with alfalfa seeds, and it was observed that it was not possible to reduce the numbers of salmonellae and *E. coli* O157:H<sup>-</sup> by more than 5log cfu/g without affecting the germination [19, 29–31]. Wuytack et al. [32] used high hydrostatic pressure as the sole physical treatment step to reduce the numbers of pathogens on radish seeds by more than 6log, however the germination rate dropped to less than 30%. In our experiments, the numbers of *E. coli* O157:H<sup>-</sup> were reduced on all seeds by more than 5log cfu/g at temperatures above 58 °C, whereas 55 °C brought about a reduction of just less than 5log cfu/g. Our results indicate that a generally valid regime for hot-water treatment, which is applicable to all kinds of seeds, does not exist but that a regime needs a specific definition for each seed type, and in our work, we treated just those seeds that are of most importance for the food industry. For sprouters operating on a small scale, our studies provide an efficient, simple and cheap method to reduce the probability of the presence of pathogens that have the character of a critical control point. Furthermore, the recommendation by the NACMCF can be achieved without the use of disinfectants, which is poorly accepted by consumers preferring organic food.

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## Chapter 5

### Characterization of the microbiota of sprouts and their potential for application as protective cultures

#### Abstract

The microbiota of ten seeds and ready-to-eat sprouts produced thereof was characterized by bacteriological culture and denaturing gradient gel electrophoresis (DGGE) of amplified DNA fragments of the 16S rRNA gene. The predominant bacterial biota of hydroponically grown sprouts mainly consisted of enterobacteria, pseudomonades and lactic acid bacteria. For adzuki, alfalfa, mung bean, radish, sesame and wheat, the ratio of these bacterial groups changed strongly in the course of germination, whereas for broccoli, red cabbage, rye and green pea the ratio remained unchanged. Within the pseudomonades, *Pseudomonas gesardii* and *Pseudomonas putida* have been isolated and strains of the potentially pathogenic species *Enterobacter cancerogenes* and *Pantoea agglomerans* were found as part of the main flora on hydroponically grown sprouts. In addition to the flora of the whole seedlings, the flora of root, hypocotyl and seed leafs were examined for alfalfa, radish and mung bean sprouts. The highest and lowest total counts for aerobic bacteria were found on seed leafs and hypocotyls, respectively. On the other hand, the highest numbers for lactic acid bacteria on sprouts were found on the hypocotyl. When sprouting occurred under the agricultural conditions, e.g. in soil, the dominating flora changed from enterobacteria to pseudomonades for mung beans and alfalfa sprouts. No pathogenic enterobacteria have been isolated from these sprout types. Within the pseudomonades group, *Pseudomonas jesenii* and *Pseudomonas brassicacearum* were found as dominating species on all seedling parts from soil samples. In practical experiments, a strain of *P. jesenii* was found to exhibit a potential for use as protective culture, as it suppresses the growth of pathogenic enterobacteria on ready-to-eat sprouts.

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## Introduction

Sprouted seeds used for human consumption originate from Far East countries and have spread in the past decades to other parts of the world, including Europe and the United States. On the German market an extraordinary great variety of different types of sprouts can be found including those from the following seeds: adzuki bean (*Phaseolus angularis*), alfalfa (*Medicago sativa*), broccoli (*Brassica oleracea* convar. *botrytis*), cress (*Lepidium sativum*), lentil (*Lens culinaris*), mung bean (*Phaseolus aureus*), mustard (*Sinapis alba*), green and yellow pea (*Pisum sativum*), onion (*Allium cepa*), radish (*Raphanus sativus*), rice (*Oryza sativa* L.), rye (*Secale cereale*), sesame (*Sesamum indicum*), sunflower (*Helianthus annuus*) and wheat (*Triticum aestivum*) [48]. Most popular are alfalfa, mung beans and radish. Spouts are produced in hydroponic culture by soaking the seeds in water followed by incubation in a warm, humid environment to optimize germination and sprout growth. The common sprouting conditions (2-7 days of sprouting, temperatures of 20-40°C and optimum water activity) are also favourable for bacterial growth [40]. At the first day of sprouting the total bacterial counts increase by 2 to 3 log units and attain a maximum level of about  $10^6$ - $10^9$  cfu/g after 2 days [19]. Seeds commonly contain high microbial loads, ranging between  $10^3$  to  $10^6$  cfu/g which are constituted mainly of pseudomonades, enterobacteria and lactic acid bacteria [32, 37, 33]. Although seed sprouts have a 'healthy' image [11, 15] they had been involved in numerous outbreaks of foodborne diseases. Most of them had been traced back to seeds contaminated with enterobacteria, e.g. salmonella and *Escherichia coli* O157:H7 [28, 40, 18, 38, 39, 13, 51]. The largest outbreak was associated with the consumption of radish sprouts involving >6000 persons in Japan [46]. The potential growth of human pathogens such as salmonella and *E. coli* O157:H7 in these microbial communities is of major concern.

To prevent the growth of pathogens on seeds and sprouts, it is necessary to understand the development of the indigenous flora during sprouting. The majority of studies have focussed on the incidence of food pathogens [32, 4, 16, 23, 33]. On the other hand, little is known about the microbial succession occurring in the chain expanding from seed to packed sprouts and furthermore differences in the microbial associations present on different sprout parts such as hypocotyl, seed leaves and roots. Because pseudomonades and enterobacteria had been found to constitute the main flora on sprouts [4, 33], these bacterial groups are especially competitive on seeds and sprouts under the conditions of industrial production. In food, enterobacteria are in their majority considered as undesirable organisms as several species are pathogenic or potentially pathogenic and have already caused several outbreaks in association with sprout consumption [26, 46, 18, 51]. Several studies have shown that *Pseudomonas* sp.

have a beneficial effect e.g. as growth promoter of plants [22], as producer of antifungal or bacteriocin-like compounds [29, 34, 6, 30]. In nature, pseudomonades are ubiquitous and an especially great variety of strains were found in soil [24, 36]. When it is intended to use competitive bacteria as protective cultures, pseudomonades are good candidates provided they exhibit an antagonistic effect against pathogenic bacteria.

In this communication we report on the characterization of the microbiota which develops in the course of germination of seeds to ready-to-eat sprouts. For mung bean and radish sprouts, the microbiota of seedling parts of the sprouts grown hydroponically in soil was studied by using the PCR-DGGE and bacteriological culture combined with 16S rRNA sequence analysis. Dominating *Pseudomonas* strains were isolated and investigated for their ability to compete with the contaminating flora during germination, e.g. salmonella.

## Materials and Methods

### Bacterial strains and counting, growth conditions

Total bacterial counts, counts of enterobacteria and pseudomonades were determined using Plate count (PC), VRBD and GSP agar (Merck), respectively, and aerobic incubation at 30°C. Lactic acid bacteria (LAB) were cultivated on MRS agar [9] under modified atmosphere (2% O<sub>2</sub>, 10% CO<sub>2</sub>, 88% N<sub>2</sub>) at 30°C. *Pseudomonas jessenii* LTH 5930 was isolated from sprouts and routinely grown at 30°C in S1 broth (containing per liter: 15 g tryptone, 3 g yeast extract, 6 g NaCl and 1 g glucose [pH 7.5]). To prepare the inocula for the challenge experiments with mung bean seeds, *Salmonella* Senftenberg LTH5703 was grown overnight at 37°C in S1 broth and shaking at 200 rpm. To determine the counts of salmonella on seeds and sprouts, plant material homogenates in saline-tryptone solution (containing per liter: 8.5 g NaCl and 1.0 g tryptone [pH 6.0]) were plated on bismuth sulphite agar (Merck) and agar plates were incubated at 37 °C for 48 h.

### Germination conditions and sampling

Seeds were provided by a German sprout producer (Deiters und Florin, Hamburg, Germany). To approach industrial germination conditions, sprouts were grown hydroponically at 30°C in sterile glass petri dishes. Depending of seed type 10-20 g of seeds were placed on a moistened filter paper, and every 24 h water was added to ensure sufficient moisture. For sprouting in soil, 1.5-4 g of seeds were distributed in the soil of flower pots covered with a layer of 0.5-1 cm of soil. Water was added to provide an  $a_w$  value > 0.99 during germination at 30°C. Every 24 h samples were taken until the germination was finished. To eliminate adherent soil,

sprouts were washed 5 times by shaking with sterile water in a sterile glass flask. Thereafter, 5-10 g of seeds, sprout parts or whole sprouts were added to a 10fold volume of saline-tryptone (containing per liter: 8.5 g NaCl and 1.0 g tryptone [pH 6.0]). The dilutions were subjected to plating on the different agar plates.

### **Recovery of isolates and bacterial mass**

From agar plates on which the highest dilution was plated (30 to 300 colonies), 6-10 colonies were picked taking the colony type into consideration. The isolates obtained from PC, VRBD and GSP agar and from MRS agar were purified using PC agar and MRS agar, respectively. Colonies of purified isolates were resuspended in sterile bidest. water to obtain a so called milky solution (MS) [7] which was used for PCR-DGGE analysis. Alternatively, from this agar plates the bacterial biomass was harvested with a sterile spreader using 4 ml of saline-tryptone. This resuspended bacteria biomass (RBB) was stored at -20°C and used for PCR-DGGE analysis.

### **DNA extractions**

After thawing of frozen samples on ice, the total DNA from RBB was extracted as described by Wang et al. [45] with modifications. Briefly, after washing of the cells, the pellet was resuspended in 100 µl of lysis buffer (6.7 % sucrose, 50 mM Tris HCl [pH 8.0], 10 mM EDTA, 20 mg lysozyme per ml, 1000 U mutanolysin per ml, 100 µg RNaseA per ml). After incubation for 1 h at 37°C, 6 µl of SDS (20 %) and 5 µl of proteinase K solution (15 mg/ml) were added and the mixture was further incubated for 30-60 min at 60°C until the cells lysed. After cooling on ice, 350 µl of Tris HCL [pH 8.0] were added and the mixture was extracted once with 500 µl phenol/chloroform/isoamyl alcohol [25:24:1] and twice with chloroform. After ethanol precipitation the DNA was dissolved in 50 µl Tris HCl [pH 8.0]. DNA from milky solution was isolated using the High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals) with the following modification. Incubation in presence of lysozyme was extended to 60 min.

### **PCR amplification**

Amplification was carried out using a Thermocycler Primus 96 plus (MWG Biotech) and universal primers HDA1GC and HDA2 [43]. The reaction mixture (50 µl) contained 5 pmol of each primer, 10 mM of each deoxyribonucleotide triphosphate, reaction buffer, 0.5 µl *rTaq* polymerase (Genaxxon) and 1 µl of DNA solution. The amplification programme was 94°C

for 4 min; 33 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s; and finally 72°C for 7 min. For species identification of pure cultures the 16S rRNA gene was amplified using primers 616V (5'-AGAGTTTGATYMTGGCTC-3') and 630R (5'-AAGGAGGTCATCCARCC-3').

### **DGGE and excision of DNA fragments**

DGGE was performed as described previously by Walter [44] with the following modification: the gel contained a 32.5 to 40% gradient of urea and formamide increasing in the direction of electrophoresis. Excision and purification of DNA fragments from DGGE gels were performed as described by Ben Omar and Ampe [5].

### **Sequence analysis**

DNA sequences of PCR fragments obtained from pure cultures or from purified DGGE bands were determined by using the SequiTherm EXCEL II DNA sequencing kit (Biozyme) LICOR system (LICOR) and the primer 609V (5'-TTAGATACCCTMGTAGT-3') or HDA2, respectively. To determine the closest relatives of the partial 16S rDNA sequences, a search of GeneBank DNA database was conducted by using the BLAST algorithm [2]. A similarity of > 97% to 16S rRNA gene sequences of type strains was used as the criterion for identification.

### **Bacteriocin screening test**

Using the bacteriocin screening test described by Fleming et al. [17] strains of *P. jessenii* and *P. brassicacearum* isolated from radish and mung bean sprouts grown in soil were studied for their ability to inhibit foodborne pathogens. Briefly, 1 µl of an overnight culture of each strain was spotted onto the surface on a bacteriocin screenin agar [41] and grown for 24h at 30°C. A top layer of 7 ml softagar (containing per liter: 2 g glucose and 7 g agar [pH 7]) inoculated with 0.14 ml of an overnight culture of the indicator strain was poured onto the bottom layer. After 16 h of incubation, bacteriocin producers were detected by the formation of inhibition zones. The indicator strains were as follows: *Listeria innocua* LTH3096, *Bacillus subtilis* LTH451, *Staphylococcus aureus* LHT906, *Enterobacter cloacae* LTH5221, *Salmonella* Typhimurium LTH781. Besides these, the pseudomonas strains were tested for their antagonistic activity against each other.

### Challenge experiments with *P. jessenii* LTH5930

Before inoculation with bacteria, mung bean seeds were decontaminated by hot water treatment (70°C for 5 min) as described previously by Weiss and Hammes [47]. In series A of the experiments, mung bean seeds were contaminated with *Salmonella* Senftenberg LTH5703, to obtain a density of  $10^2$ - $10^3$  cfu/g on dry seeds. To do this, cells of overnight cultures of *S. Senftenberg* LTH5703 were washed with sterile water, resuspended in 10 ml sterile water and added to 1 kg of mung bean seeds by mixing for 1 min. The drying and storage of the contaminated seeds was performed as described previously by Weiss and Hammes [47]. These contaminated seeds were further inoculated by an overnight culture of *P. jessenii* LTH 5930. Again, cells were harvested by centrifugation, washed with sterile water and mixed with contaminated seeds to obtain a density of  $10^8$  cfu/g. Samples without *P. jessenii* served as control. In series B, mung bean seeds (not contaminated with salmonella) were inoculated with an overnight culture of *P. jessenii* (see series A) to obtain a density of  $10^8$  cfu/g on the seeds. After germination for 1 d, sprouts were inoculated with *Salmonella* Senftenberg LTH5703 (see series A) to obtain a density of 1-10 cfu/g on the sprouts.

## Results

### Bacterial counts on hydroponically grown sprouts

To get insight into the bacterial load of adzuki, alfalfa, broccoli, red cabbage, green pea, radish, rye, sesame and wheat sprouts, the development of total counts as well as counts of enterobacteria, pseudomonades and lactic acid bacteria (LAB) were monitored up to 6 days of germination of the seeds. The results are compiled in Table 1. The aerobic total bacterial counts of the seeds (day 0) varied markedly in a range of below the detection limit of 2 log cfu/g (green pea) up to 6.3 log cfu/g (rye). After 24 h of germination, the total counts increased by 2-3 log units. At the end of germination, the lowest load of bacteria was 7.90 log cfu/g for green pea, whereas the highest was found on sesame sprouts with 9.51 log cfu/g.

For broccoli, red cabbage and green pea, the proportion of bacterial groups did not change during germination from seeds to sprouts. For broccoli and red cabbage, pseudomonades constituted the dominant microbiota (ca. 70%), whereas the dominating biota (ca. 75%) of green pea seeds and sprouts consisted of enterobacteria. On wheat sprouts, mainly enterobacteria were found at the first day of germination but after 2 days pseudomonades became dominant (75-80%). Also on rye seeds, enterobacteria were found to be the predominating bacteria (ca. 80%) after 1 day of germination, but their counts decreased to ca. 58% of the total bacterial counts at the end of germination. Pseudomonades constitute the



main part (84%) of bacteria on sesame seeds, however after one day of germination, the biota changed and enterobacteria became dominant on the sesame sprouts.

### **Characterisation of the microbiota by bacteriological culture**

For a more detailed characterisation of the microbiota on sprouts, mung bean and radish seeds, because of their practical relevance, were germinated under hydroponic conditions and, for the purpose of comparison, in soil. Total counts and counts of enterobacteria, pseudomonades and LAB of the seeds and the sprouts as well as of their roots, hypocotyls, and cotyledons were monitored. The results are compiled in Table 2. Bacterial counts of mung bean seeds were under the limit of detection whereas radish seeds contained more than 5 log cfu/g. During germination of mung beans in soil, the dominating biota on the different parts of the sprouts consisted of pseudomonades (88-95%), followed by enterobacteria (2-19%) and LAB (1-6%). The highest load of enterobacteria (19%) was found on the hypocotyl part after 48h. When mung beans were grown hydroponically, the dominating bacteria were enterobacteria, whereas pseudomonades and LAB played a minor role only. After 24h of germination the different parts consisted of 90-98% enterobacteria, followed by 2-10% LAB and 1-2% of pseudomonades. At the end of germination, the part of pseudomonades increased up to 17-19% on whole sprouts, roots and hypocotyl, whereas on seed leaves only 2% pseudomonades have been found. The highest percentage of lactic acid bacteria (12%) were found on the hypocotyl after 48h. During germination of radish in soil, the dominating biota on the different parts of the sprouts consisted of pseudomonades (90-93%), followed by enterobacteria (2-16%) and LAB (2-8%). The highest load of LAB (47%) was found on the hypocotyl part after 48h. When mung beans were grown hydroponically, the different plant parts consisted of 45-50% pseudomonades, 30-44% of enterobacteria and 1-20 % of lactic acid bacteria. The highest percentage of LAB (36%) was found on the hypocotyl part.

**Table 1** Bacterial counts of hydroponically grown sprouts determined on PC (total bacterial counts), VRBD (enterobacteria), GSP (pseudomonades) and MRS (lactic acid bacteria) agar

Seed type	Cell counts* (log cfu/g) after															
	0 h				24 h				48 h				72h			
	PC	VRBD	GSP	MRS	PC	VRBD	GSP	MRS	PC	VRBD	GSP	MRS	PC	VRBD	GSP	MRS
Adzuki	2.98 ± 0.28	<2 ± 0.00	<2 ± 0.00	<2 ± 0.00	6.62 ± 0.15	6.28 ± 0.15	5.56 ± 0.15	3.75 ± 0.14	7.82 ± 0.05	7.55 ± 0.15	6.52 ± 0.14	4.16 ± 0.09	8.70 ± 0.10	7.87 ± 0.19	7.31 ± 0.08	4.64 ± 0.15
Brokkoli	4.26 ± 0.20	2.88 ± 0.30	3.32 ± 0.17	<2 ± 0.00	7.38 ± 0.70	6.86 ± 0.42	6.66 ± 0.16	6.23 ± 0.15	8.78 ± 0.02	8.21 ± 0.05	8.62 ± 0.02	6.41 ± 0.43	8.74 ± 0.04	8.49 ± 0.03	8.73 ± 0.01	6.53 ± 0.26
Green pea	<2 ± 0.00	<2 ± 0.00	<2 ± 0.00	<2 ± 0.00	7.69 ± 0.14	7.21 ± 1.81	6.06 ± 0.07	5.98 ± 0.29	7.75 ± 0.03	7.55 ± 0.03	7.03 ± 0.09	5.82 ± 0.17	7.90 ± 0.14	7.75 ± 0.04	7.30 ± 0.37	6.23 ± 0.05
Red cabbage	3.32 ± 0.25	<2 ± 0.00	<2 ± 0.00	<2 ± 0.00	7.52 ± 0.00	5.70 ± 0.43	6.06 ± 0.17	5.57 ± 0.15	8.54 ± 1.42	7.49 ± 0.15	7.96 ± 0.19	5.66 ± 0.04	9.21 ± 0.07	8.66 ± 0.20	9.02 ± 0.13	5.96 ± 0.01
Rye	6.29 ± 0.21	6.04 ± 0.03	5.56 ± 0.09	<2 ± 0.00	8.73 ± 0.72	8.39 ± 0.04	7.79 ± 0.11	5.59 ± 0.07	8.72 ± 0.06	8.46 ± 0.10	8.46 ± 0.04	6.50 ± 0.13	8.84 ± 0.02	8.60 ± 0.07	8.37 ± 0.02	6.85 ± 0.15
Sesame	4.22 ± 0.07	<2 ± 0.00	3.04 ± 0.11	<2 ± 0.00	7.91 ± 0.04	7.51 ± 0.07	6.26 ± 0.07	5.88 ± 0.61	8.08 ± 0.24	7.33 ± 0.18	7.13 ± 0.04	5.81 ± 0.35	9.51 ± 0.12	8.05 ± 0.04	7.33 ± 0.01	6.20 ± 0.16
Wheat	5.89 ± 0.00	5.51 ± 0.33	5.40 ± 0.00	<2 ± 0.00	8.16 ± 0.03	7.83 ± 0.08	7.50 ± 0.42	5.37 ± 0.02	7.95 ± 1.17	7.60 ± 0.70	8.08 ± 0.17	5.13 ± 1.58	8.33 ± 0.23	7.61 ± 0.80	8.20 ± 0.07	4.39 ± 0.35

\* Mean values and standard deviation of three independent experiments

**Table 2** Bacterial counts of radish and mung bean sprouts, grown hydroponically or in soil, and of the different parts thereof during germination. Counts were determined on PC (total bacterial counts), VRBD (enterobacteria), GSP (pseudomonades) and MRS (lactic acid bacteria) agar

Growth conditions	Seed type	Cell counts* (log cfu/g) after											
		0 h				24 h				48 h			
		PC	VRBD	GSP	MRS	PC	VRBD	GSP	MRS	PC	VRBD	GSP	MRS
No Soil	Mung bean												
	Whole sprout / Seed	2.5	< 2,0	< 1,0	<1,0	6.9 ± 0.1	6.3 ± 0.2	3.5 ± 0.3	4.6 ± 0.3	7.1 ± 0.4	6.4 ± 0.1	5.8 ± 0.0	4.0 ± 0.2
	Root	n.a.	n.a.	n.a.	n.a.	6.2 ± 0.1	5.8 ± 0.1	4.1 ± 0.1	4.0 ± 0.1	7.5 ± 0.2	6.9 ± 0.2	6.2 ± 0.2	3.8 ± 0.1
	Hypocotyl	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5.5 ± 0.2	5.0 ± 0.0	4.4 ± 0.1	4.3 ± 0.2
	Cotyledons	n.a.	n.a.	n.a.	n.a.	6.6 ± 0.1	5.4 ± 0.3	2.2 ± 0.2	4.4 ± 0.2	7.4 ± 0.1	7.0 ± 0.1	5.3 ± 0.2	4.6 ± 0.2
	Radish												
	Whole sprout / Seed	5.5	3.9	5.2	2.0	7.9 ± 0.5	7.5 ± 0.3	7.4 ± 0.3	6.7 ± 0.6	8.0 ± 0.4	7.4 ± 0.7	7.7 ± 0.1	7.2 ± 0.2
	Root	n.a.	n.a.	n.a.	n.a.	7.0 ± 1.0	6.7 ± 0.7	6.7 ± 0.3	6.2 ± 0.5	8.0 ± 0.6	7.4 ± 0.5	7.8 ± 0.9	7.3 ± 0.5
	Hypocotyl	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	7.2 ± 0.5	6.6 ± 0.7	7.0 ± 0.2	6.9 ± 0.0
	Cotyledons	n.a.	n.a.	n.a.	n.a.	7.8 ± 0.5	7.4 ± 0.3	7.4 ± 0.7	6.9 ± 0.1	7.9 ± 0.7	7.2 ± 0.4	7.6 ± 0.2	7.0 ± 1.5
Soil	Mung bean												
	Whole sprout / Seed	2.5	< 2,00	< 1,00	<1,00	6.5 ± 0.1	5.4 ± 0.3	6.4 ± 0.1	4.9 ± 0.5	5.8 ± 0.0	4.0 ± 0.1	5.6 ± 0.2	3.7 ± 0.1
	Root	n.a.	n.a.	n.a.	n.a.	5.8 ± 0.1	3.8 ± 1.3	5.3 ± 0.1	3.6 ± 0.6	6.4 ± 0.1	4.2 ± 0.4	5.7 ± 0.4	3.9 ± 0.4
	Hypocotyl	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5.1 ± 0.0	4.3 ± 0.2	4.9 ± 0.2	3.8 ± 0.2
	Cotyledons	n.a.	n.a.	n.a.	n.a.	6.1 ± 0.2	4.9 ± 0.0	5.9 ± 0.2	4.5 ± 0.3	6.3 ± 0.1	4.5 ± 0.1	6.2 ± 0.1	4.5 ± 0.0
	Radish												
	Whole sprout / Seed	5.5	3.9	5.2	2.0	7.2 ± 0.3	6.3 ± 0.0	7.0 ± 0.1	5.7 ± 0.1	6.8 ± 0.5	4.9 ± 0.0	6.6 ± 0.5	5.3 ± 0.0
	Root	n.a.	n.a.	n.a.	n.a.	6.4 ± 0.2	4.9 ± 0.0	6.1 ± 0.0	4.9 ± 0.0	6.6 ± 0.4	4.9 ± 0.2	6.3 ± 0.1	5.9 ± 0.2
	Hypocotyl	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	5.7 ± 0.1	4.3 ± 0.1	5.2 ± 0.0	5.3 ± 0.1
	Cotyledons	n.a.	n.a.	n.a.	n.a.	7.0 ± 0.4	6.0 ± 0.1	6.9 ± 0.3	5.9 ± 0.2	7.3 ± 0.4	6.7 ± 0.2	7.1 ± 0.2	6.8 ± 0.0

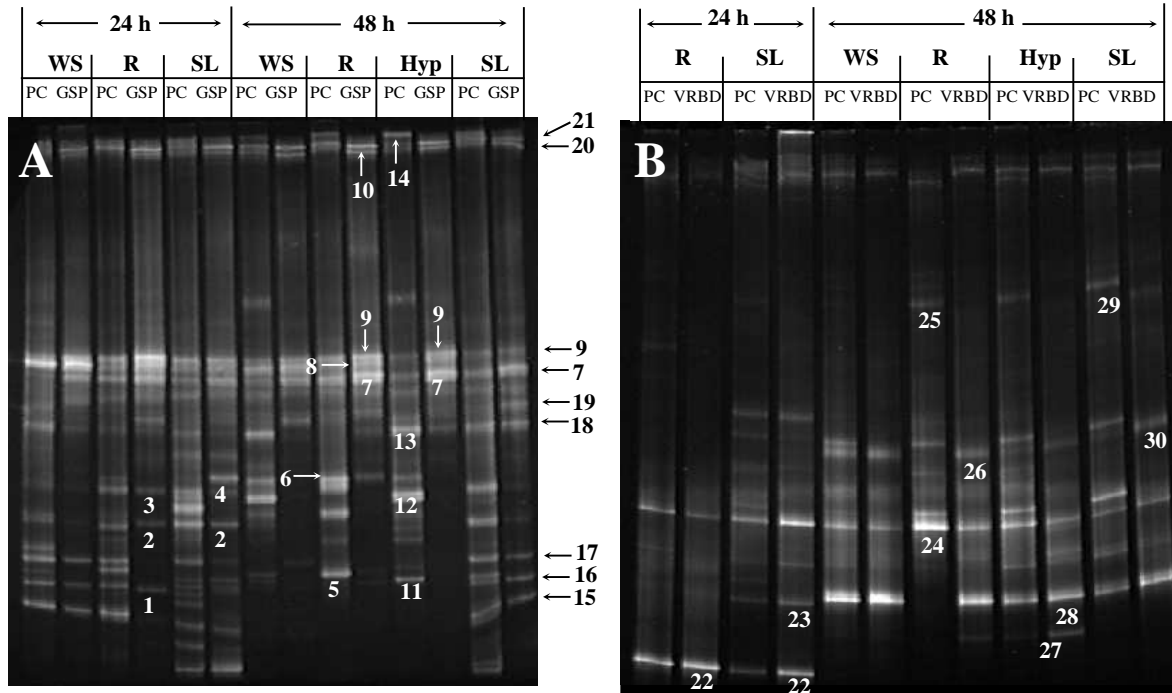
\* Mean values and standard deviation of three independent experiments

n.a. = not applicable, seedling part was not developed

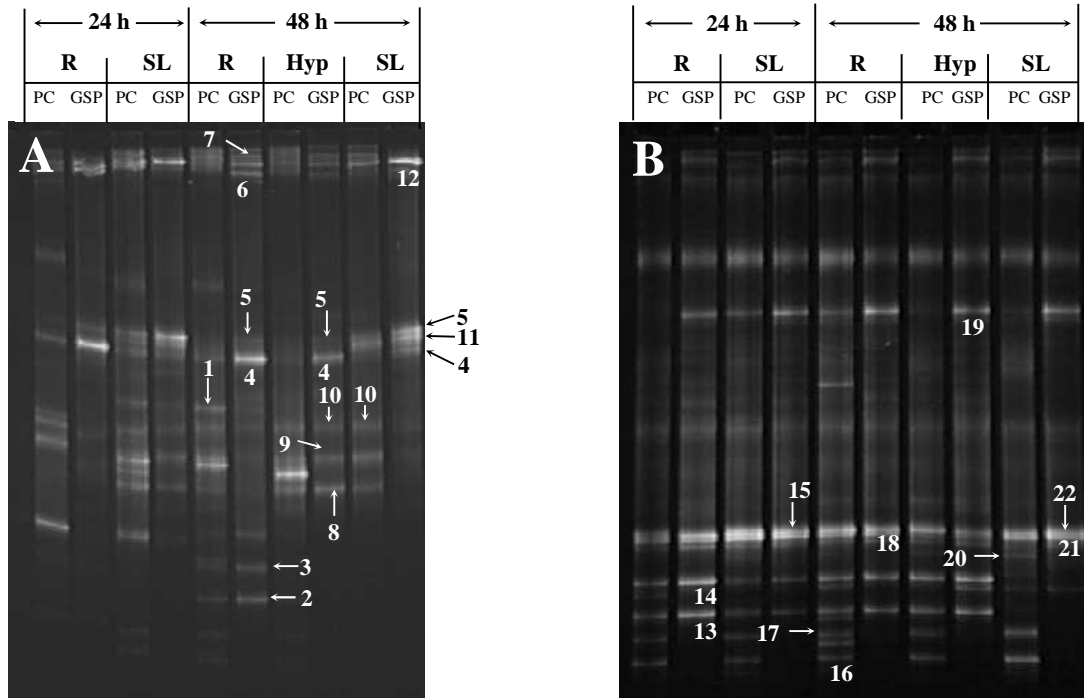
### Characterization of the microbiota by using PCR-DGGE

To determine the components of the dominating microbiota up to the genus or species level, PCR-DGGE analyses was performed using 16S rRNA gene targeted universal primers and DNA isolated from the RBBs obtained from PC and GSP agar plates. The DGGE profiles of PCR products obtained from mung bean sprouts grown in soil are shown in Fig. 1A. Profiles obtained from RBB after 24 h of germination compared with those after 48 h indicated a decrease in the complexity of the bacterial community. For example, bands 1 to 3, obtained from the root after 24 h as well as bands 4 and 5, obtained from seed leaves after 24h, were not present anymore after 48 h of germination. With regard to the different seedling parts, the bacterial community of seed leaves showed the highest complexity. Major bands obtained from the roots after 48 h as well as from seed leaves could mainly be allotted to *Pseudomonas* species, whereas those obtained from the hypocotyl were allotted to *Bacillus* spec. Among the pseudomonades, *P. jessenii* and *P. brassicacearum* were found to be dominant on all sprouting parts after 24 and 48 h of germination. Strains of these species were used in a screening for potential candidates for protective cultures. The DGGE profiles of PCR products obtained from hydroponically grown mung bean sprouts are shown in Fig. 1B. The profiles are less complex compared to those obtained from mung bean sprouts grown in soil, but the complexity of the microbiota also decreased during germination. Bands 22 and 23, obtained after 24h were not present anymore after 48 h of germination. As main components of the microbiota of the hydroponically grown mung bean sprouts, species of the genus *Bacillus*, *Enterobacter* as well as *Azotobacter beijerinckii* were identified.

DGGE profiles of PCR products obtained from radish sprouts grown in soil are shown in Fig. 2A. The profiles obtained were less complex as those obtained from mung bean sprouts grown in soil. On the roots mainly pseudomonades and isolates of the genera *Bacillus* and *Serratia* could be identified. On the hypocotyl and seed leaves pseudomonades and strains of the group *Raoultella/Klyvera* were found. Among the pseudomonades, again the species *P. jessenii* and *P. brassicacearum* dominated. Therefore, strains of these species were again subjected to the protective culture screening. The DGGE profiles of PCR products obtained from radish sprouts grown hydroponically are shown in Fig. 2B. The microbiota markedly differed from that of the radish sprouts grown in soil. The bands obtained from RBB of PC agar were mainly allotted to the genus *Xenorhabdus* or the groups *Escherichia vulneris/fergusonii* and *Enterobacter cowanii/cancerogenus*.



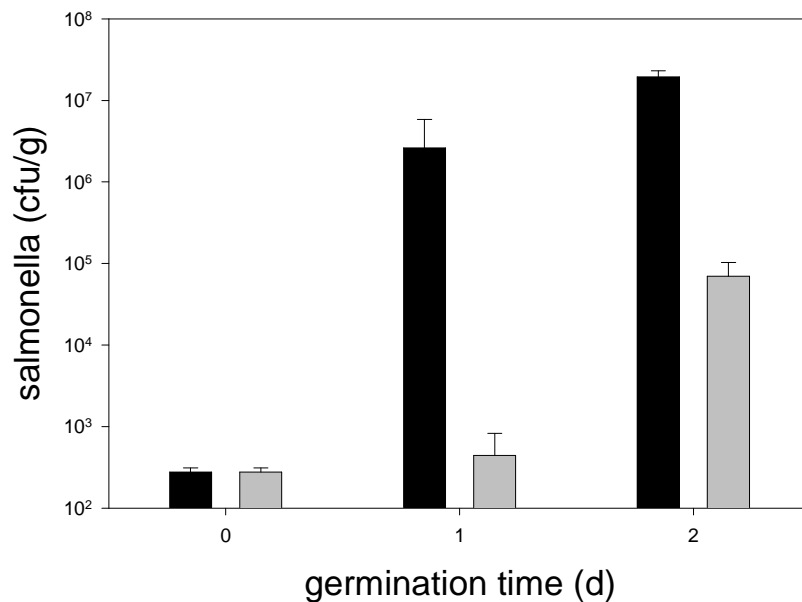
**Figure 1.** DGGE profiles of PCR products obtained with primer pair HDA1GC/HDA2 and DNA isolated from the RBBs of mung bean sprouts, grown in soil (A) or hydroponically (B), and parts thereof. RBBs were obtained from whole sprouts (WS), roots (R), seed leaves (SL) or the hypocotyle (Hyp) upon culture on PC, GSP and VRBD agar plates. Based on 16S rDNA sequence analyses, the isolates were identified as follows: 1, *Comamonas testosteroni*; 2 and 3, *Raoultella* sp.; 4, *Pseudomonas graminis*/*Pseudomonas flavescens*; 5, *Vogesella indigofera*; 6, 10 and 20, *Pseudomonas* sp.; 11 and 26 *Bacillus* sp.; 12, *Bacillus fastidiosus*; 13, *Bacillus simplex*; 14 and 30, *Bacillus flexus*/*Bacillus megaterium*; 15, 16 and 17, *Serratia* sp.; 18, *Pseudomonas fragi*; 19, *Pseudomonas syringae*; 21, *Pseudomonas viridiflava*; 22, *Pantoea agglomerans*/*Enterobacter asburia*; 23 and 25, *Enterobacter* sp.; 24, *Azotobacter beijerinckii*; 27 and 28, *Xenorhabdus* sp.; 29, *Enterobacter dissolvens*/*Enterobacter cloacae*. Based on comparison of the PCR-fragment migration distance with the patterns of the isolates, the fragments were allotted to the following species: 7, *Pseudomonas brassicacearum*; 8 and 9, *Pseudomonas jessenii*.



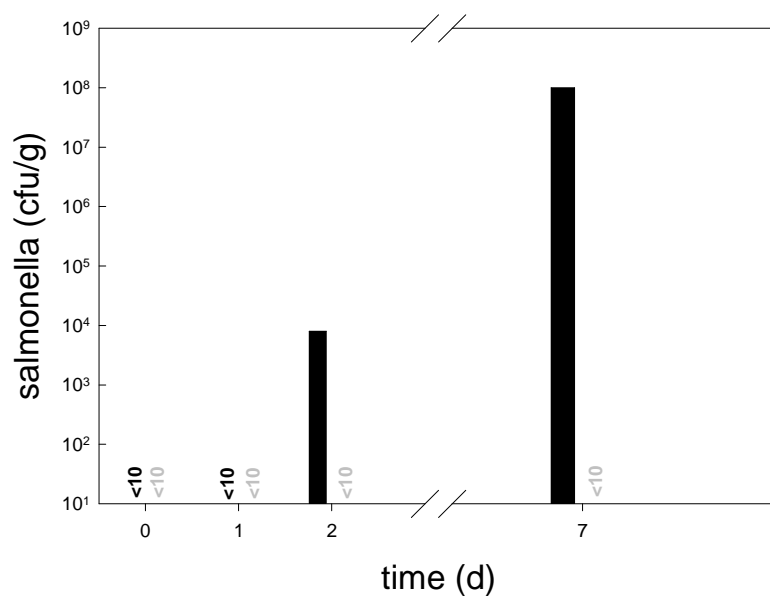
**Figure 2.** DGGE profiles of PCR products obtained with primer pair HDA1GC/HDA2 and DNA isolated from the RBBs of parts of the radish sprouts, grown in soil (A) or hydroponically (B). RBBs were obtained from roots (R), seed leaves (SL) or the hypocotyle (Hyp) upon cultured on PC and GSP agar plates. Based on 16S rDNA sequence analyses, the isolates were identified as follows: 1 and 7, *Bacillus sp.*; 2, 3 and 15, *Serratia sp.*; 6, *Pseudomonas viridiflava*; 8, 9 and 10, *Raoultella sp.*; 12, *Pseudomonas sp.*; 13 *Xenorhabdus sp.*; 14, *Escherichia vulneris/Escherichia fergusonii*. Based on comparison of the PCR-fragment migration distance with the patterns of the isolates, the fragments were allotted to the following species: 4, *Pseudomonas brassicacearum*; 5 and 11, *Pseudomonas jessenii*; 16 and 17, *Enterobacter cowanii*; 18, *Pantoea agglomerans/ Enterobacter cancerogenes*; 19, *Pseudomonas gessardii*; 20 and 21, *Pseudomonas sp.*; 22, *Enterobacter cancerogenes*.

### Use of *Pseudomonas* strains in protective culture

The characterization of the microbiota of sprouts revealed that strains of the species *P. jessenii* and *P. brassicacearum* predominated on sprouts grown in soil. Due to their high capability to compete with the naturally occurring microbiota, some strains were further investigated for their potential use in protective culture. Using the bacteriocin screening test, 4 strains of *P. jessenii* and 2 strains of *P. brassicacearum* were investigated for their potential to inhibit growth of model organisms for foodborne infections as well as each other. Among all strains, *P. jessenii* LTH5930 was the most suitable strain (data not shown), as it exhibited activity against *S. aureus*, *B. subtilis*, *S. Typhimurium*, *Enterobacter cloacae* and all the other pseudomonas strains. Furthermore, strain LTH 5930 showed the highest antagonistic activity against *E. cloacae* and *L. innocua*. Therefore, strain *P. jessenii* LTH593 was selected to be used in a protective culture and challenge experiments (series A and B) were performed using *S. Senftenberg* as indicator organism. In series I, *P. jessenii* ( $10^8$  cfu/g) was added to mung bean seeds contaminated with salmonella and growth of both bacteria was monitored during the hydroponical germination. When compared with control without pseudomonades, salmonella showed a reduced growth on the sprouts which resulted in cell count of  $>3$  log and  $>2$  log cfu/g below the control after 24 and 48h incubation, respectively (Fig. 3). In series B seeds were pre-inoculated with  $10^8$  cfu/g *P. jessenii* LTH5930 and thereafter low contamination levels (1-10 cfu/g) of salmonella (added at day 1) were added. Remarkably, under these conditions salmonella were not able to grow during germination whereas in the control without pseudomonades salmonella grew up to counts of  $10^7$  cfu/g (Fig. 4).



**Figure 3.** Development of the cell counts of salmonella during germination of hydroponically grown mung bean sprouts with (■) and without (■) the use of *Pseudomonas jessenii* LTH5930 as protective culture.



**Figure 4.** Development of the cell counts of salmonella during germination of hydroponically grown mung bean sprouts with (■) and without (■) the use of *Pseudomonas jessenii* LTH5930 as protective culture.

## Discussion

The characterization of the microbiota of sprouts grown hydroponically revealed that, for certain kinds of seeds, the bacterial composition may totally change during germination from seed to sprout, whereas for other kinds the proportion of the main groups of bacteria on ready to eat sprouts is the same as found on seeds. These type of studies have not been performed before as other publications of sprouts focussed mainly on the presence of pathogens or, when analysing the microbial load, the investigation did not differentiate between enterobacteria and pseudomonades [32, 4, 33, 25].

PCR-DGGE has been demonstrated to be a suitable tool to characterise the dynamics of the microbial population in several ecosystems, e.g. sourdough [27], faeces [44], rhizosphere [12, 35]. In these studies, bacterial DNA was isolated directly from the ecosystem, whereas in the present study it was not feasible to amplify the DNA from sprouts by using PCR. This phenomenon has already been observed in studies of Dent et al. [10], showing that plant DNA and other ingredients of plant origin interfere with PCR. To circumvent that obstacle, we used the DNA extracted from RBB, as we have already shown that PCR-DGGE of RBB provides results that are consistent with that of culture technique and is therefore a rapid and reliable method to characterize the culturable living part of a microbiota [7].

It is striking that the composition of the flora and the proportion of the three main groups of bacteria at the end of germination is similar within plant families. Sprouts from the *Fabaceae* (mung bean, adzuki bean and green pea) contained mainly enterobacteria, whereas those of *Brassicaceae* (broccoli, radish and red cabbage) after germination harboured pseudomonades together with enterobacteria. However, this relationship between plant families and the bacterial groups has not been found to occur on the corresponding seeds. Therefore, the microbial load on the seeds does not necessarily affect the composition of the main bacteria on hydroponically grown sprouts. This finding differs greatly from the results obtained with soil grown sprouts. On these sprouts, pseudomonades rather than enterobacteria became the main flora of sprouts. Species of the genera *Pseudomonades*, *Arthrobacter*, *Clostridium*, *Achromobacter*, *Micrococcus*, *Flavobacterium* and *Bacillus* are the mainly isolated soil bacteria [21]. Pseudomonades from soil itself are known for their potential to adhere on special plant parts such as the roots [20] and, remarkably, Pierson et al. [31], Weller [49] and Whipps [50] have already studied the potential of pseudomonades acting as biological protective agents against plant diseases. In our study, the findings of Gisi et al. [20] have been confirmed, and, furthermore, it was shown that pseudomonades became the predominant



bacteria on all plant parts of mung bean and radish sprouts grown in soil. Remarkably, these pseudomonades were found to inhibit the growth of enterobacteria.

Pseudomonades are possible candidates for use as protective cultures, because they belong to the German L1 risk group [3], except *Pseudomonas aeruginosa*, a known nosocomal pathogen [8]. Pseudomonades comprise furthermore plant pathogens and pectinolytic strains [1, 14], affecting the quality of plant products. For *P. jesseni* this potential has not been reported [42]. The use of isolate *P. jessenii* LTH5930 as protective has the potential to improve food safety as it suppresses the growth of salmonella which, together with other enterobacteria, constitute the main cause of food born infections due to the consumption of sprouts.

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## Chapter 6

### **Lactic acid bacteria as protective cultures against *Listeria* spp. on cold-smoked salmon**

#### **Abstract**

Three bacteriocin producing (Bac<sup>+</sup>) strains of *Lactobacillus sakei* were used singly and in combination with each other as protective cultures to control the growth of listeria in cold-smoked salmon. Challenge experiments were conducted under practical conditions in a smokehouse. The surface of salmon sides was inoculated with 10<sup>4</sup> cfu/g of *Listeria innocua* and 10<sup>7</sup> cfu/g of Bac<sup>+</sup> lactic acid bacteria as well as a *L. sakei* Bac<sup>-</sup> control. After smoking the counts of listeria and lactic acid bacteria were determined at days 1 and 14. All Bac<sup>+</sup> *L. sakei* strains reduced the counts of *L. innocua* by > 2 log units. Strain LTH5754 was an isolate from cold-smoked salmon and achieved even a 5 log reduction of *L. innocua* within the storage period. *In vitro* experiments showed, that the Bac<sup>+</sup> strains were also effective against *L. monocytogenes* (3 strains tested) and *L. ivanovii* (1 strain). The pH as well the sensorial properties of the smoked salmon were not affected by the *L. sakei* inocula.



## Introduction

Cold-smoked salmon is usually stored at  $< 5^{\circ}\text{C}$  under vacuum or in modified-atmosphere. Under these conditions certain human pathogens [1] can grow among which *Listeria monocytogenes* has become of special concern as several studies have shown that it is not uncommon to detect *Listeria monocytogenes* in cold-smoked salmon [2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]. This pathogen originates from contaminated fish as well as from the processing environments [13, 14, 15], and its numbers may increase in the course of cold storage [16, 17, 18, 19, 20]. To protect the consumers, the FDA of the U.S. and certain European countries (e.g. France, Austria and Italy) imposed a zero tolerance, whereas others (e.g. Germany, Sweden and Denmark) advocate less than 100 cfu/g at the sell-by date [21]. To improve food safety, preservatives such as sodium nitrate and sodium lactate have been tested for their efficiency in reducing levels of *Listeria monocytogenes* [22, 23, 24]. However, the application of preservatives interferes either with the sensory properties or is not accepted. As an alternative process biopreservation with cultures of lactic acid bacteria (LAB) [25, 26, 27, 28, 29, 30] and/ or their antagonistic products have been studied for an antilisterial efficiency [31, 32, 33, 34, 35]. These studies were conducted at laboratory scale with cold-smoked salmon slices or water/salmon suspensions as model systems. The aim of our study was to investigate the efficiency of three  $\text{Bac}^+$  strains of *Lactobacillus sakei* in reducing the numbers of listeria on cold-smoked salmon under the practical conditions in a smokehouse.

## Materials and Methods

### Microorganisms and culture conditions

In preliminary experiments 6 strains of lactic acid bacteria (LAB) were screened for their anti-listeria effect on cold-smoked salmon. We used 4 bacteriocin producing ( $\text{Bac}^+$ ) strains of *L. sakei*, one strain of each *L. curvatus* ( $\text{Bac}^+$ ) and *L. reuteri* (a producer of reutericyclin [36]) and *L. sakei* LTH681 ( $\text{Bac}^-$ ) as a control. *Listeria innocua* LTH3096 was used as the challenge organism. As further strains were used: *L. ivanovii* LTH3097, *L. monocytogenes* (type strain DSMZ 20600) as well as *L. monocytogenes* LTH 4593 (Serotype 4) and *L. monocytogenes* LTH 4593 (Serotype 4b, kindly provided by L. Axelsson, Matforsk, Norwegian Food Research Institute, Ås, Norway). *Listeria* were grown at  $30^{\circ}\text{C}$  in S1 broth containing the following per liter: tryptone (15g), yeast extract (3g), sodium chloride (6g) and glucose (1g). The pH was adjusted to 7.5. LAB were grown in MRS-broth at  $37^{\circ}\text{C}$ . The cells were harvested by centrifugation and washed twice with sterile peptone saline. A suspension

was made from the pellet containing 20% milk powder and 5% glycerol and stored at  $-80^{\circ}\text{C}$ . Microbial counts were determined after serial diluting of samples, surface plating on MRS agar containing 0.1 % brominecresolgreen (LAB) and Palcam agar (listeria), respectively. Listeria counts of  $<10^2$  cfu/g were determined by using the most-probable-number (MPN)-method with Fraser broth (Merck). LAB were tested for their anti-listeria activity with the aid of the agar-drop-test [37].

### **Preparation and inoculation of salmon juice**

Sterile water was added to fresh salmon at a ratio of 5:1, followed by homogenization using a stomacher. At lower ratios the emulsion appeared very stiff and might not have permitted a homogeneous distribution of the inocula. This suspension was inoculated with  $10^4$  cfu/ml of *Listeria* spp. After one hour LAB were added to obtain a density of  $10^7$  cfu/ml.

### **Smokehouse experiments**

Freshly prepared salmon sides were surface inoculated before smoking with ca.  $10^4$  cfu/g of *L. innocua* and  $10^7$  cfu/g of LAB contained in peptone saline. For that purpose each salmon side was weighed, the required cell counts calculated and the salmon rubbed by hand with the various cultures. After smoking in the smoking-chamber at ca.  $25^{\circ}\text{C}$  for 10 hours, the samples were vacuum packed and stored at  $4^{\circ}\text{C}$ . At days 1 and 14 the counts of listeria and LAB were determined. A sensory evaluation of odour and taste was performed by 5 experts at days 1 and 14 after production. In addition, the pH-value was measured with an electrode pH21 (Schott, Germany). The mean of three measurements were recorded.

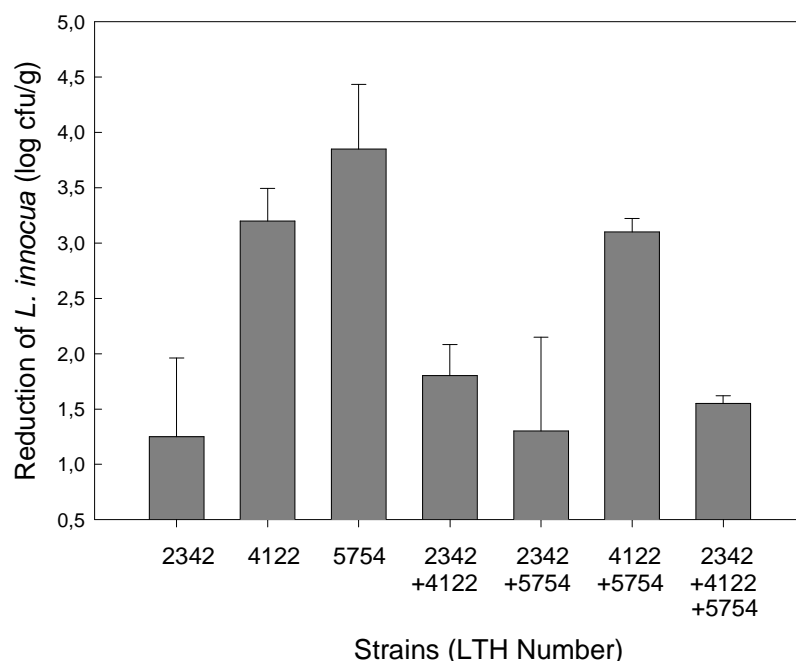
## **Results**

### **Screening the anti-listerial activity of LAB**

It was confirmed that all selected  $\text{Bac}^+$  strains inhibited *L. innocua* in an agar drop test (data not shown). In the first series of smokehouse experiments, these strains were studied further for their potential to inhibit *L. innocua* on salmon sides. After storage for 14 days at  $4^{\circ}\text{C}$ , it was observed that strains *L. sakei* LTH2342, LTH4122 and LTH5754 reduced *L. innocua* by  $>1$  log when compared with the effect of the control  $\text{Bac}^-$  strain (LTH681). In this control as well as in a further one without LAB-inoculum, the counts of *L. innocua* remained at the level of the inoculum ( $10^4$  cfu/g). These three most effective *L. sakei* strains were used in further experiments.

### Inhibition of *L. innocua* on salmon sides

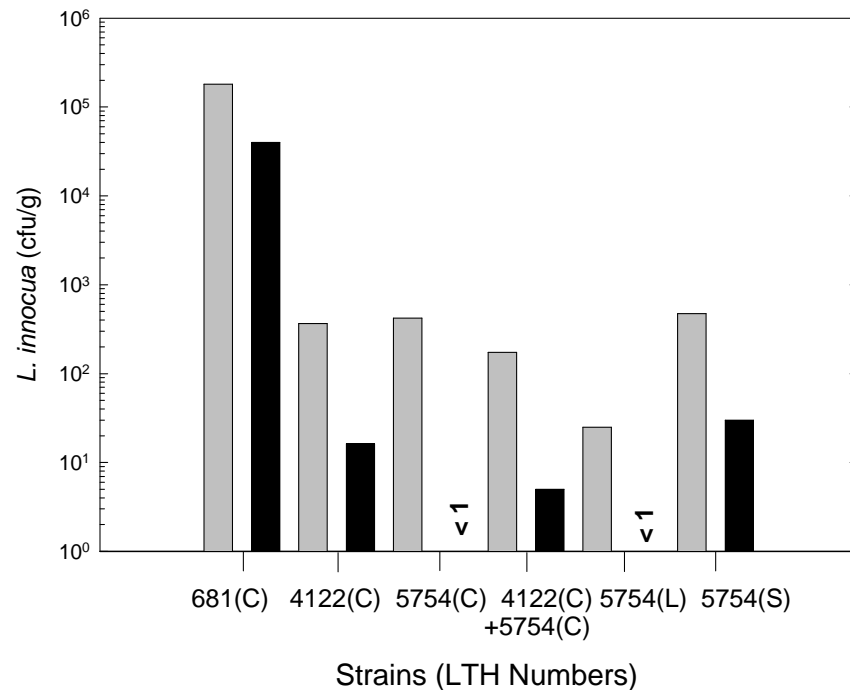
With strains *L. sakei* LTH2342, LTH4122 and LTH 5754, each employed singly as well as in combination, challenge experiments were performed with salmon sides pre-contaminated with *L. innocua*. Each experiment was repeated at least three times. It was verified in the agar drop test that none of the Bac<sup>+</sup> LAB strains inhibited the other. In Fig. 1 the effect on the *L. innocua* counts of the singly applied strains and their combinations is depicted. The values are normalized in relation to the effect of a bacteriocin negative control. Strain LTH2342 used singly or in combination with the other strains reduced the numbers of *L. innocua* by 1-2 log cfu/g. Strain LTH4122 brought about a >3 log reduction, and the fish isolate LTH5754 achieved even >3.5 log during the storage period of 14 days. The counts for LAB remained in the range of the inoculum of 10<sup>6</sup>-10<sup>7</sup> cfu/g. The combination of LTH5754 and LTH4122 reduced listeria by > 3 log but did not enhance the effect of the singly used strains. With strain LTH4122 and LTH5754 further investigations were performed.



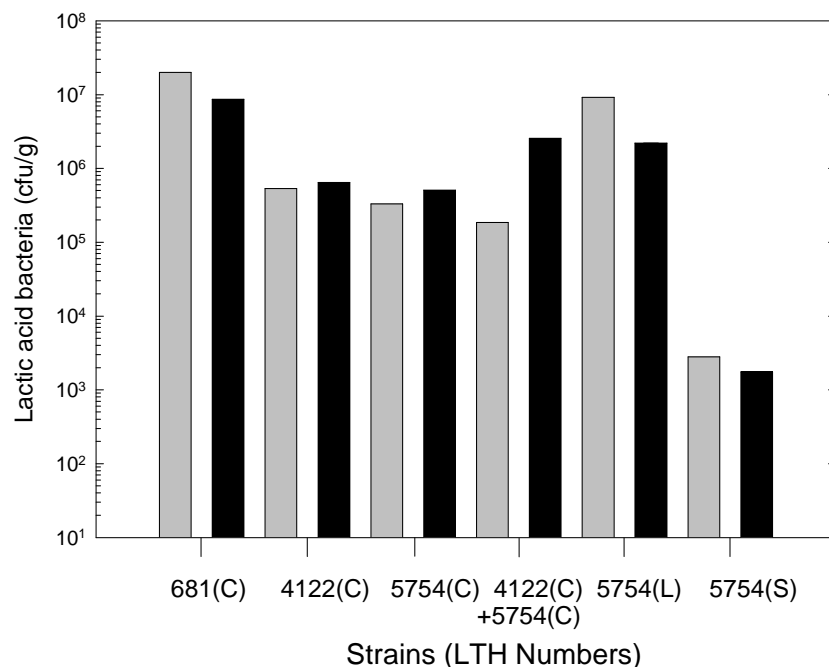
**Fig. 1** Effect of Bac<sup>+</sup> strains and their combinations on the reduction of *L. innocua* determined at day 14. The values are normalized in relation to the effect of a bacteriocin negative control

Three further experimental series were performed, and in Fig. 2 the results of one representative series are depicted. At day 1 strains LTH4122 and 5754 as well as the

combination thereof reduced the counts of *L. innocua* by  $>2.5$  log. At day 14 the listeria counts were reduced by  $>3$  log with LTH4122 and even by 5 log with *L. sakei* 5754. We used also a lyophilized preparation of *L. sakei* LTH5754 as an inoculum. This preparation was adjusted before surface application to obtain a density of  $10^7$  cfu/g of salmon. The results are included in Fig. 2 and show that the effect is comparable with that obtained with the frozen cultures. In addition the effect of the filter sterilised supernatant of an overnight culture of *L. sakei* LTH5754 was studied (Fig. 2). At day 1 the effect on *L. innocua* was comparable with the reduction observed with strain inocula of LTH4122 and LTH5754. However, at day 14, the cell free supernatant brought about a reduction of 3 log only. To ascertain that the LAB in the inoculum remain in the active state, their numbers were determined. As shown in Fig. 3, the LAB counts were in the range of  $5 \times 10^5$ - $10^7$  cfu/g until day 14. The counts of LAB after treatment with the cell free supernatant were ca.  $10^3$  cfu/g.



**Fig. 2** The effect of cultures (C), lyophilized cultures (L) and culture supernatant (S), respectively, on the reduction of *L. innocua* on salmon determined at day 1 (■) and day 14 (■) of storage at 4°C.



**Fig. 3** Lactic acid bacteria counts determined on salmon during storage at day 1 (■) and day 14 (■) after application of cultures (C), lyophilized cultures (L) and culture supernatant (S), respectively.

The sensory evaluation at day 1 and day 14 revealed that the high cell counts of the LAB inocula had no negative effect on the odour and taste of the cold-smoked salmon. The pH at the end of the storage period was in the range of 6.1-6.3 for all samples and showed no difference between the samples with and without LAB inoculum.

### **Inhibition of *Listeria* spp. in salmon juice**

As under the conditions of industrial practice pathogens cannot be used in challenge experiments, we performed *in vitro* experiments in order to show that various strains of *L. monocytogenes* are also inhibited by the LAB activity, i. e. that *L. innocua* is an adequate model, as it had been described by several authors [38, 39]. Cold-smoked salmon juice was used as a model substrate and strains of three strains of *L. monocytogenes* were used as target inocula. It was observed with *L. sakei* LTH4122 and *L. sakei* LTH5754 that within 24h of incubation at 5 °C the counts of all *Listeria* strains were reduced by ca. 2 log units, whereas their numbers increased by 2 orders of magnitude in the controls without Bac<sup>+</sup> LAB. The LAB numbers remained at the level of the inoculum (10<sup>7</sup> cfu/ml).

## Discussion

The results demonstrate the potential of *L. sakei* Bac<sup>+</sup> strains for their use as protective cultures with activity against *Listeria* spp. under the conditions of industrial production of vacuum packed cold-smoked salmon stored at 4°C. In our investigations the experiments were performed in a smokehouse and therefore studies with *L. monocytogenes* as challenge organism were not feasible. Agar drop tests and experiments with salmon juice as model system confirmed that the LAB (Bac<sup>+</sup>) strains were also effective against strains of *L. invanovii* and *L. monocytogenes* including those of serotype 4b. These findings are consistent with the observations of Hugas [38] and Steeg [39] showing that the inhibition of *L. innocua* is indicative for a corresponding effect against *L. monocytogenes*. In our studies we inoculated the salmon sides just before smoking. Under these conditions the LAB were able to adapt to the fish habitat, to multiply and to express their anti-listerial activity within the smoking period of 10 hours at 28°C. This favorable environment may explain the strong reducing effect on *L. innocua* observed already after one day of storage. *L. sakei* LTH5754 is an isolate obtained from cold-smoked salmon which was produced in the very smokehouse, and surpassed the reduction effect of strain LTH4122 by 1 log. Practical application of bacteriocin producing organism in food protection is still rather scarce. Indeed, the preconditions for the successful use of these types of cultures are demanding. For example the organism have to be metabolically active, have to produce the bacteriocin and, as it has been shown by Gänzle [40], the antibacterial activity of the bacteriocin itself depends on the prevailing environmental conditions. Our practical experiments have shown that this preconditions were fulfilled and the anti-listerial effect became clearly evident. It was, furthermore, observed that the use of the LAB (Bac<sup>+</sup>) culture as inoculum is more efficient than the application of a culture supernatant that contains the antagonistic activity. Finally, for the practical use of the Bac<sup>+</sup>-strains it is of advantage that they can be transformed in a lyophilised preparation and keep their full antagonistic potential.

The sensorial evaluation as well the pH-values at the end of storage have shown that the inoculation of the salmon sides with LAB did not affect the sensory properties of the cold-smoked salmon. Therefore the application of *L. sakei* LTH4122 and *L. sakei* 5754 have the potential for improving the safety of cold-smoked fish.

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## Chapter 7

### Concluding remarks

Fore these studies, two hypotheses for improved food safety were formulated and have been shown to be valid. The first hypothesis included that the application of a physical together with a biological treatment is an efficient way to improve the food safety status of raw, ready-to-eat sprouts. The second hypothesis included the use of an anti-listerial protective culture on cold smoked salmon, efficient when applied under the conditions of the practice in a smokehouse.

According to the Codex Alimentarius Commission (CAC, 2001), Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP) are prerequisites to achieve the adequate food safety status of RTE fruits and vegetables. In the code of “Hygienic Practices for Fresh Fruits and Vegetables” which includes in the Annex related to “Ready-to-eat Fresh Pre-cut Fruits and Vegetables”. For these the following factors were elaborated, which affect the microbiological safety of the products: The hygienic conditions related to handlers, environment, storage, transport, cleaning, sanitation and production facilities with respect to the quality of water, treatment of manure and soil, the use of agricultural chemicals, biological control as well as indoor facilities. Furthermore, an important element is the application of the concept of Hazard Analysis and Critical Control Points. The concept permits a systematic approach to the identification of hazards and an assessment of the likelihood of their occurrence during the manufacture, distribution and use of a food product, and defines measures for their control (ILSI, 2004).

When focussing on sprout manufacturing, the assurance of the absence of pathogens is the ultimate aim of a Critical Control Point in the production process. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1999) recommended as a process objective a 5 log units reduction of pathogens on seeds as a means of safety control in the production process. This committee found on a sound scientific base: Quantitative analyses performed on seeds associated with disease upon consumption of sprouts revealed that the numbers of pathogens ranged between 1 and 6/100 g of seeds. Therefore, the worst case scenario for seed contamination was assumed to be 1 pathogen/10 g of seeds. It was also assumed that 50 kg of seeds is the amount of starting material for each batch of sprouts. This

leads to a calculation of a number of 5000 pathogens per batch of sprouts and thus a 5 log treatment will yield 0.05 pathogens/batch.

In the US it was found that exposure of the seeds to a concentration of 20.000 mg/kg of calcium hypochlorite achieves basically this level of decontamination and this treatment became a recommended measure and a critical control point (FDA, 2001). There has been so far no other seed treatment applied in practice, which achieved the recommendation of the NACMCF. Our studies have shown that the heat treatment of the different kinds of seeds is as effective as the application of calcium hypochlorite in reducing the pathogens (e.g. salmonellae and EHEC) that are mainly involved in outbreaks due to the consumption of contaminated sprouts (Chapter 3 and 4). In some cases the pathogens on the seed had been reduced by even more than 7 log units with the aid of a hot water treatment. In so far, the hot water treatment is superior to the chlorine treatment which conclusion is supported by two publications. Thomas et al. (2003) studied industrial practices and compliance with U.S. FDA guidelines among California Sprout Firms. It was observed that producers of alfalfa, clover and radish sprouts achieved the FDA-recommended decontamination levels (based on the NACMCF recommendation), whereas producers of mung bean sprouts do not achieve the recommended level of 20.000 ppm calcium hypochlorite. In another study, performed by Proctor et al. (2001) it had been observed that the required level of free chlorine had not been achieved on alfalfa and this has apparently caused the multistate *Salmonella* outbreak. The study showed that even on alfalfa seeds the recommended level of free chlorine had not been achieved. As the use of disinfectants for the production of organic food is poorly accepted (at least in Germany), its application in Europe is not allowed when producing according to organic food legislation (EWG, 1991). It has been verified that our studies, performed with mung bean seeds, led to the application of hot water treatment by the market leader for sprouts in Germany (N. Deiters, 2006; Company Deiters and Florin, Hamburg, Germany; personal communication).

In the European Union, process objectives for sprouts have not been defined. In the recent Commission Regulation EC 2073/2005, the following food safety criteria for sprouts have been set: Absence of *Salmonella* in 25 g with  $n=5$ ,  $c=0$ . The group criterion of *Listeria monocytogenes* does also apply:  $<100$  cfu/g at the end of shelf life. Furthermore the process hygiene criterion was set: *E. coli*,  $n=5$ ,  $c=2$ ,  $m=100$  cfu/g,  $M=100$  cfu/g. Clearly, the scientific basis is as good as established in the NACMF approach and it is left to the processor to work in agreement with the set criterion.

To ensure the safety of sprouts during the manufacturing process, the use of protective cultures as biological preservation method constitutes a safety hurdle to prevent the growth of pathogens in cases when the sprouts become contaminated during germination process, which is consistent with the assumption that a lack of GMP had taken place.

Protective cultures have already shown their potential for application in various foods that include raw meat (Hugas, 1998; Vermeiren et al., 2004), ready-to-eat meals (Rodgers, 2001), seafood (Brillet et al., 2004; Alves et al., 2005; Ghalfi et al., 2006) and increasingly fruits and vegetables (Bennik et al., 1999; Janisiewicz et al., 1999; Liao and Fett, 2001; Wei et al., 2006). Basically new is the use of components of the natural microbiota for protective cultures. In our studies (Chapter 5) it was shown, that the biota of ready to eat sprouts when grown hydroponically is completely different to the flora of sprouts that had been grown in soil. Our results indicated the existence of soil biota components, which are able to compete with the natural seed flora. As one of the main components we isolated *Pseudomonas jessenii* LTH5930. This strain was obtained from mung bean as well as radish sprouts grown in soil (identified as the same strain on the bases of molecularbiological techniques). When applied from the very beginning of germination of mung bean seeds, this strain became the predominant biotic element of the flora. Our results showed that truly competitive organism almost exclusively can be found in soil. Furthermore this strain had the ability to adapt to the conditions of the hydroponical germination. Strain LTH5930 was used in challenge experiments to inhibit the growth of salmonellae and was effective in *in vivo* experiments with germinating mung beans. It was shown that a contamination of the seeds with salmonellae during the production process can be prevented by the use of *Pseudomonas jessenii* LTH5930.

As lactic acid bacteria have been used for several thousands of years within food fermentation processes, they have the status of GRAS (generally recognized as safe) organism in the US. Their application as starter or protective culture e.g. in the meat industry therefore is commonly applied and well accepted. However, for other microorganisms with limited familiarity, their safety has to be proven. In April 2005, the Scientific Committee of the EU has evaluated on request of EFSA related to “the generic approach to the safety assessment of microorganisms used in food/feed and the production of food/feed additives” (request No EFSA-Q-2004-021). In this approach, the qualified presumption of safety (QPS) - presumption being defined as „an assumption based on reasonable evidence“; qualified to allow certain restrictions to apply - is discussed. According to EFSA, QPS would provide a qualified generic approval system that would harmonise the safety assessment of

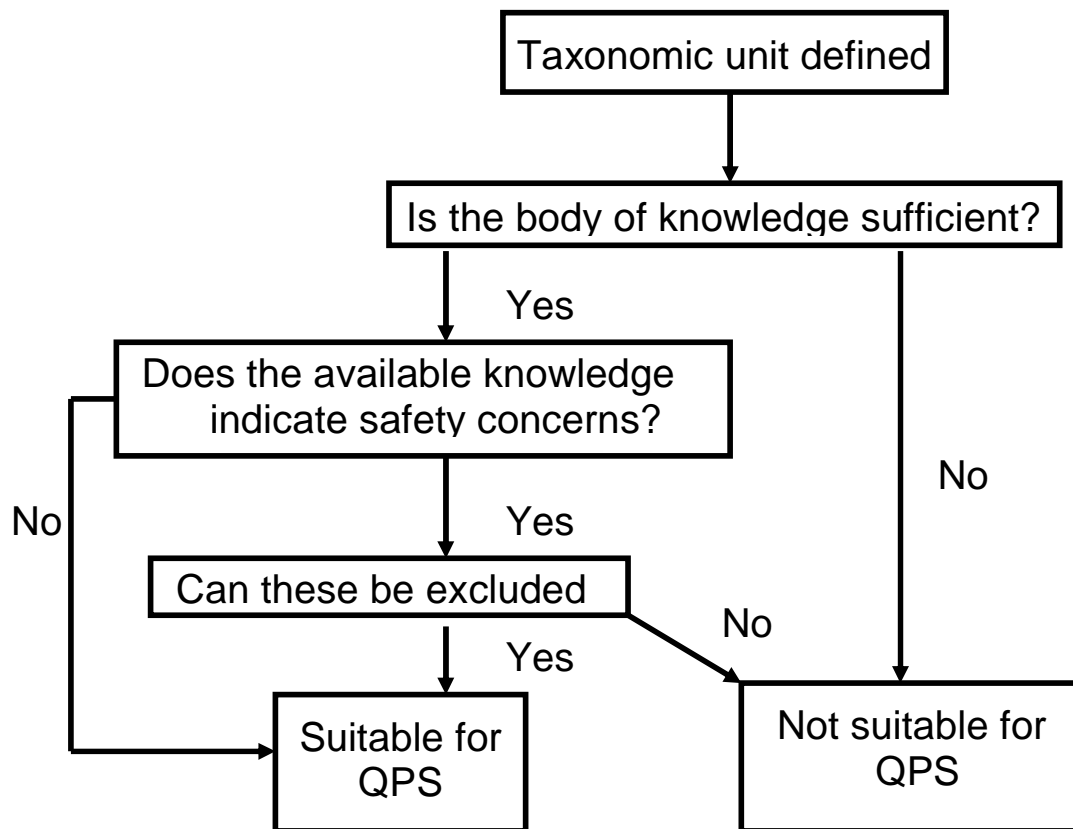
microorganisms throughout the food chain. This could be done without either compromising the standards set for microorganism used in animal feeding stuffs or requiring all organism used in food production with a long history of use to be subjected to a full and unnecessary safety review. Thereafter it would aid the consistency of assessment and make better use of assessments resources without compromising safety. A case-by-case safety assessment then could be limited to only those aspects that are relevant for the organism in question (e.g. presence of acquired antibiotic resistance, determinants in a lactic acid bacterium or known virulence factors in a species known to contain pathogenic strains). Establishing QPS status, as originally proposed, rested in four pillars:

- 1) Taxonomy - the taxonomic level of grouping for which QPS is sought
- 2) Familiarity - whether sufficient is known about the proposed group of microorganism to reach a decision on their safety
- 3) Pathogenicity- whether the grouping considered for QPS contains known pathogens, if so whether sufficient is known about their virulence determinants of toxigenic potential to exclude pathogenic strains
- 4) End use - whether viable organisms enter the food chain or whether they are used to produce other products

The QPS status would be determined in advance of any specific safety assessment, and products/processes involving organisms not considered suitable for QPS would not be excluded but would remain subject to a full safety assessment. In Figure 1, a generalized scheme for assessing the suitability for QPS status of microorganism is shown. When following the QPS recommendation, it is obvious that the use of *Pseudomonas* strains as protective culture, the QPS status has still to be determined in advance.

The ensuring of food safety of smoked salmon with cultures of anti-listerial lactic acid bacteria (LAB) and/or their antagonistic products have been investigated in many studies. These studies were conducted at laboratory scale with cold-smoked salmon slices or water/salmon suspensions as model systems. So far, no protective cultures have been studied and their efficiency against *Listeria* spp. tested under the practical conditions of a smokehouse. In our studies (Chapter 6), the protective cultures we used had a very strong effect after 24 h, moreover it was a continuous elasting effect during the storage of the smoked salmon until the end of shelf-life. The knowledge of the process parameters is very important for the assurance of the effectivity of the protective microorganisms. As each smokehouse has its own manufacturing conditions (e.g. skinning, washing procedures, salting as well as smoking conditions regarding time and temperature, packaging and storage etc.), it

is essential to know the process parameters to adapt the microorganisms to the process conditions. For example, in one experiment, a mixture of organic acids was used, to minimize the native load of bacteria, before we inoculated the fish with our protective cultures and the listeria, respectively. Under these conditions the LAB cultures were not able to inhibit the growth of listeria. Furthermore, we also conducted experiments in another smokehouse that has already tested anti-listerial protective organisms from a commercial supplier. According to the culture supplier the preparation had already shown positive effects on gravad salmon.



**Figure 1** Generalised scheme for assessing the suitability for QPS status of microorganisms



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## Chapter 8

### Summary

The safety of raw, ready-to-eat foods is of paramount importance and is in the focus of the food industry, consumers as well as food scientists. To improve the food safety status of the products, efficient decontamination as an important processing step and/or the use of protective microorganisms as biocontrol agents are promising approaches. In our work we successfully used these approaches for raw sprouts and cold-smoked salmon as examples for RTE foods. Therefore the set goals have been successfully performed and essential scientific knowledge has been contributed. The results have been published and are described in the following in form of the respective abstracts.

#### **Thermal seed treatment to improve the food safety status of sprouts**

The use of chemicals to reduce microbial contaminations on raw materials for the production of organic food such as sprout seeds is not allowed in Germany. To develop an alternative decontamination procedure, we studied the effect of hot water at various time/temperature regimes. Mung bean seeds were inoculated with  $>10^8$  cfu/g *Salmonella* Senftenberg W775 by immersion. This strain is known for its unusual high heat resistance. The seeds were dried and stored at 2 °C. The salmonella counts on the dried seeds remained unchanged during storage for 8 weeks. The contaminated seeds were treated at 55, 58 and 60 °C for 0.5-16 min. D-values of 3.9, 1.9 and 0.6 min, respectively, were determined and a z-value of 6.2 ( $r^2 = 0.99$ ) was calculated for the inactivation of *S. Senftenberg* W775 on the mung bean seeds. The thermal treatment at time/temperature regimes of 55°C/20 min, 60°C/10 min, 70°C/5 min and 80°C/2 min reduced the pathogens on the mung bean seeds by  $>5$  log units without affecting the germination rate of the seeds.

(Alexander Weiss and Walter P. Hammes. 2003. Thermal seed treatment to improve the food safety status of sprouts. *Journal of Applied Botany*. 77: 152-155)

### **Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H<sup>-</sup> on alfalfa, mung bean and radish seeds used for sprout production**

We studied the effect of hot-water treatment at various time/temperature regimes to design a decontamination process which is consistent with the recommendation of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) to reduce pathogens on seeds by 5log cfu/g. Alfalfa, mung bean and radish seeds were inoculated by immersion with more than 10<sup>7</sup> cfu/g of enterobacteria (*Salmonella* Senftenberg W775, *S. Bovismorbificans* and *Escherichia coli* O157:H<sup>-</sup>), dried and stored at 2 °C. The numbers of salmonellae and *E. coli* O157:H<sup>-</sup> on these seeds remained unchanged during storage for 8 weeks. To achieve sprouting rates of more than 95%, time-temperature regimes were defined. The thermal treatment of contaminated mung bean (2–20 min for 55–80 °C), radish and alfalfa seeds 0.5–8 min (53–64 °C) reduced all pathogens by more than 5log cfu/g. For *S. Senftenberg* W775 on radish seeds, D values of 3.2, 1.9 and 0.6 min were determined for exposure at 53, 55 and 58 °C and a z value of 6.2 °C was calculated. For alfalfa seeds, the respective D values were 3.0, 1.6, and 0.4 min and the z value was the same as that determined for radish seeds.

(Weiss Alexander and Hammes, Walter P. 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H<sup>-</sup> on alfalfa, mung bean and radish seeds used for sprout production. Eur. Food Res. Tech. 211, 187-191)

### **Characterization of the microbiota of sprouts and their potential for application as protective cultures**

The microbiota of ten seeds and ready-to-eat sprouts produced thereof was characterized by bacteriological culture and denaturing gradient gel electrophoresis (DGGE) of amplified DNA fragments of the 16S rRNA gene. The predominant bacterial biota of hydroponically grown sprouts mainly consisted of enterobacteria, pseudomonades and lactic acid bacteria. For adzuki, alfalfa, mung bean, radish, sesame and wheat, the ratio of these bacterial groups changed strongly in the course of germination, whereas for broccoli, red cabbage, rye and green pea the ratio remained unchanged. Within the pseudomonades, *Pseudomonas gesardii* and *Pseudomonas putida* have been isolated and strains of the potentially pathogenic species *Enterobacter cancerogenes* and *Pantoea agglomerans* were found as part of the main flora on hydroponically grown sprouts. In addition to the flora of the whole seedlings, the flora of root, hypocotyl and seed leaves were examined for alfalfa, radish and mung bean sprouts. The

highest and lowest total counts for aerobic bacteria were found on seed leafs and hypocotyls, respectively. On the other hand, the highest numbers for lactic acid bacteria on sprouts were found on the hypocotyl. When sprouting occurred under the agricultural conditions, e.g. in soil, the dominating flora changed from enterobacteria to pseudomonades for mung beans and alfalfa sprouts. No pathogenic enterobacteria have been isolated from these sprout types. Within the pseudomonades group, *Pseudomonas jessenii* and *Pseudomonas brassicacearum* were found as dominating species on all seedling parts from soil samples. In practical experiments, a strain of *P. jessenii* was found to exhibit a potential for use as protective culture, as it suppresses the growth of pathogenic enterobacteria on ready-to-eat sprouts.

(Alexander Weiss, Christian Hertel, Silke Grothe, Diep Ha and Walter P. Hammes. 2006. Characterization of the microbiota of sprouts and their potential for application as protective cultures. System. Appl. Microbiol. Submitted for publication)

#### **Lactic acid bacteria as protective cultures against *Listeria* spp.on cold-smoked salmon**

Three bacteriocin producing (Bac<sup>+</sup>) strains of *Lactobacillus sakei* were used singly and in combination with each other as protective cultures to control the growth of listeria in cold-smoked salmon. Challenge experiments were conducted under practical conditions in a smokehouse. The surface of salmon sides was inoculated with 10<sup>4</sup> cfu/g of *Listeria innocua* and 10<sup>7</sup> cfu/g of Bac<sup>+</sup> lactic acid bacteria as well as a *L. sakei* Bac<sup>-</sup> control. After smoking the counts of listeria and lactic acid bacteria were determined at days 1 and 14. All Bac<sup>+</sup> *L. sakei* strains reduced the counts of *L. innocua* by > 2 log units. Strain LTH5754 was an isolate from cold-smoked salmon and achieved even a 5 log reduction of *L. innocua* within the storage period. *In vitro* experiments showed that the Bac<sup>+</sup> strains were also effective against *L. monocytogenes* (3 strains tested) and *L. ivanovii* (1 strain). The pH as well the sensorial properties of the smoked salmon were not affected by the *L. sakei* inocula.

(Weiss Alexander and Hammes, Walter P. 2006. Lactic acid bacteria as protective cultures against *Listeria* spp.on cold-smoked salmon. Eur. Food Res. Tech. 222, 343-346)

## Chapter 9

### Zusammenfassung

Die Lebensmittelsicherheit von rohen, verzehrsfertigen Lebensmitteln ist von überragender Bedeutung was Lebensmittelherstellern, Verbrauchern sowie Lebensmittelwissenschaftlern bewusst ist. Um den Status der Lebensmittelsicherheit zu verbessern, ist die Dekontamination durch Waschen mit heissem Wasser ein wichtiger Prozessschritt; hinzu kann der Einsatz von Schutzkulturen als biologisch wirksames Agends vielversprechendes Verfahren kommen. In unserer Arbeit haben wir diese Strategien erfolgreich bei roh zu verzehrenden Keimlingen und Räucherlachs angewandt. Somit wurden die gesetzten Ziele erfolgreich ausgeführt und wesentliche wissenschaftliche Erkenntnisse beigetragen. Die Ergebnisse sind publiziert und werden nachfolgend in Form der veröffentlichten Zusammenfassungen beschrieben.

### Thermische Behandlung von Saaten um den Status der Lebensmittelsicherheit zu verbessern

Der Einsatz von chemischen Agenzien zur Verhinderung mikrobieller Kontamination von Rohmaterialien für die Herstellung von biologisch erzeugten Keimlingen ist in Deutschland nicht erlaubt. Um eine alternative Dekontamination zu entwickeln, haben wir den Effekt von heissem Wasser bei unterschiedlichen Zeit-/Temperaturregimen untersucht. Mungobohnensaaten wurden mit  $>10^8$  KbE/g *Salmonella* Senftenberg W775 beim Einweichen inokuliert. Dieser Stamm ist für seine aussergewöhnliche Hitzeresistenz bekannt. Die Saaten wurden getrocknet und bei 2°C gelagert. Die Lebendkeimzahlen für die Salmonellen auf den getrockneten Saaten blieb während der Lagerung für 8 Wochen unverändert. Die kontaminierten Saaten wurden bei 55, 58 and 60 °C für 0.5-16 min behandelt. D-Werte von 3.9, 1.9 und 0.6 min wurden bestimmt und ein z-Wert von 6.2 ( $r^2=0.99$ ) wurde für die Inaktivierung von *S. Senftenberg* W775 auf Mungobohnensaaten berechnet. Die thermische Behandlung der Saaten bei Zeit-/Temperaturregimen von 55°C/20 min, 60°C/10 min, 70°C/5 min und 80°C/2 min reduzierte die Pathogenen auf Mungobohnensaaten um  $>5$  log-Einheiten ohne die Auskeimungsrate der Saaten zu beeinflussen.

(Alexander Weiss and Walter P. Hammes. 2003. Thermal seed treatment to improve the food safety status of sprouts. Journal of Applied Botany. 77: 152-155)

**Die Wirksamkeit der Hitzebehandlung zur Reduktion von Salmonellen und *Escherichia coli* O157:H<sup>-</sup> auf Luzerne-, Mungobohnen- und Rettichsaaten mit Anwendung bei der Erzeugung von Keimlingen**

Wir haben die Wirksamkeit der Behandlung mit heissem Wasser bei verschiedenen Zeit-/Temperaturregimen untersucht, um einen alternativen Dekontaminationsprozess zu entwickeln, der die Empfehlung des National Advisory Committee on Microbiological Criteria for Foods (NACMCF) erfüllt, die Pathogenen auf Saaten um 5 log KbE/g zu reduzieren. Luzerne-, Mungobohnen- und Rettichsaaten wurden mit 10<sup>7</sup> cfu/g Enterobakterien (*Salmonella* Senftenberg W775, *S. Bovismorbificans* und *Escherichia coli* O157:H<sup>-</sup>) durch Einweichen inokuliert, anschliessend getrocknet und bei 2 °C gelagert. Die Keimzahlen für Salmonellen und *E. coli* O157:H<sup>-</sup> blieben auf diesen Saaten während der Lagerung von 8 Wochen unverändert. Um Keimungsraten von mehr als 95% zu erhalten wurden unterschiedliche Zeit-/Temperaturregime definiert. Die thermische Behandlung von kontaminierten Mungobohnensaaten (2–20 min für 55–80 °C), Rettich- und Luzernesaaten 0.5–8 min (53–64 °C) reduzierte alle Pathogenen um mehr als 5 log KbE/g. Für *S. Senftenberg* W775 auf Rettichsaaten, wurden D-Werte von 3.2, 1.9 und 0.6 min für 53, 55 und 58 °C bestimmt sowie ein z-Wert von 6.2 °C berechnet. Für Luzernesaaten wurden die entsprechenden D-Werte mit 3.0, 1.6, und 0.4 min bestimmt, als z-Wert wurde derselbe errechnet wie für die Rettichsaaten.

(Weiss Alexander and Hammes, Walter P. 2005. Efficacy of heat treatment in the reduction of salmonellae and *Escherichia coli* O157:H<sup>-</sup> on alfalfa, mung bean and radish seeds used for sprout production. Eur. Food Res. Tech. 211, 187-191)



### **Charakterisierung der Mikroflora von Keimlingen und ihr Potential für die Anwendung als Schutzkultur**

Die Mikroflora zehn verschiedener Saaten sowie den verzehrsfertigen Keimlingen daraus wurden mit Hilfe kulturtechnischer Methoden sowie der DGGE der amplifizierten DNA Fragmente der 16S rRNA charakterisiert. Die dominante Mikroflora von hydroponisch gewachsenen Keimlingen bestehen hauptsächlich aus Enterobakterien, Pseudomonaden und Milchsäurebakterien. Für Adzuki, Luzerne, Mungobohne, Rettich, Sesame und Weizen änderte sich das Verhältnis dieser Bakteriengruppen während des Keimungsverlaufes stark, wohingegen für Broccoli, Rotkohl, Roggen und grüne Erbse das Verhältnis unverändert blieb. Innerhalb der Gruppe der Pseudomonaden wurden *P. gesardii* und *P. putida* isoliert, Stämme der potentiell pathogenen Spezies *Enterobacter cancerogenes* und *Pantoea agglomerans* wurden als Teil der Hauptflora auf hydroponisch gewachsenen Keimlingen gefunden. Zusätzlich zu der Flora der ganzen Keimlinge wurden die Floren von Wurzel, Hypokotyl und Keimblättern von Luzernen-, Rettich- und Mungobohnenkeimlingen untersucht. Die höchsten Zahlen für Gesamtkeime wurden auf Keimblättern gefunden, die niedrigsten auf dem Hypokotyl. Die höchsten Keimzahlen für Laktobazillen wurden dagegen auf dem Hypokotyl gefunden. Bei Keimung unter agronomischen Bedingungen, z.B. in der Erde, änderte sich die dominante Flora von Enterobakterien hin zu Pseudomonaden für Mungobohnen- und Luzernekeimlinge. Von diesen Saatensorten wurden keine pathogenen Enterobakterien isoliert. Innerhalb der Gruppe der Pseudomonaden wurden *P. jessenii* und *P. brassicacearum* als dominierende Species auf allen Keimlingsteilen von Proben aus der Erde gefunden. In praktischen Experimenten wurde ein Stamm von *P. jessenii* gefunden, der das Potential für den Einsatz als Schutzkultur besitzt, das Wachstum von pathogenen Enterobakterien auf verzehrsfertigen Keimlingen zu unterdrücken.

(Alexander Weiss, Christian Hertel, Silke Grothe, Diep Ha and Walter P. Hammes 2006. Characterization of the microbiota of sprouts and their potential for application as protective cultures. Applied and Environmental Microbiology. Submitted for publication)

**Milchsäurebakterien als Schutzkultur gegen *Listeria* spp. auf kalt geräuchertem Lachs**

Drei Bakteriozin bildende ( $Bac^+$ ) Stämme von *Lactobacillus sakei* wurden einzeln und in Kombination eingesetzt, um das Wachstum von Listerien in kalt geräuchertem Lachs zu kontrollieren. Hierfür wurden „challenge tests“ in einer Räucherei unter Praxisbedingungen ausgeführt. Die Oberfläche von Lachsseiten wurden mit  $10^4$  KbE/g *Listeria innocua* und  $10^7$  KbE/g  $Bac^+$  Milchsäurebakterien inokuliert, ein *L. sakei*  $Bac^-$  Stamm diente als Kontrolle. Nach dem Räucherprozess wurden Keimzahlen für Listerien und Milchsäurebakterien am Tag 1 und 14 bestimmt. Alle  $Bac^+$  *L. sakei* Stämme reduzierten die Keimzahlen für *L. innocua* um  $> 2$  log Einheiten. Stamm LTH5754, ein Isolat von kalt geräuchertem Lachs, erreichte sogar eine Reduktion von *L. innocua* um 5 log Einheiten während der Lagerzeit. *In vitro* Experimente zeigten, dass  $Bac^+$  Stämme ebenso gegen *L. monocytogenes* (3 getestete Stämme) sowie *L. ivanovii* (1 getesteter Stamm) wirksam waren und der pH-Wert so wie die sensorischen Eigenschaften nicht durch das Inokulieren mit *L. sakei* beeinflusst wurden.

(Weiss Alexander and Hammes, Walter P. 2006. Lactic acid bacteria as protective cultures against *Listeria* spp. on cold-smoked salmon. Eur. Food Res. Tech. 222, 343-346)

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